EO/US
PCT/IL98/00487

P[illegible]ENT COOPERATION TREA[illegible]

From the INTERNATIONAL BUREAU

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NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark
Office
Box PCT
Washington, D.C.20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing: 21 October 1999 (21.10.99)	
International application No.: PCT/IL98/00487	Applicant's or agent's file reference: 114468.2 MM
International filing date: 08 October 1998 (08.10.98)	Priority date: 10 October 1997 (10.10.97)
Applicant: EDELMAN, Meir et al	

1. The designated Office is hereby notified of its election made:

☒ in the demand filed with the International preliminary Examining Authority on:

08 March 1999 (08.03.99)

☐ in a notice effecting later election filed with the International Bureau on:

2. The election ☒ was
☐ was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland** Facsimile No.: (41-22) 740.14.35	Authorized officer: J. Zahra Telephone No.: (41-22) 338.83.38

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WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ^{6} :
C12N 15/82, 5/04, A01H 5/00

A1

(11) International Publication Number: **WO 99/19498**

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(22) International Filing Date: 8 October 1998 (08.10.98)

(30) Priority Data:
PCT/IL97/00328 10 October 1997 (10.10.97) IL

(71) Applicant *(for all designated States except US)*: YEDA RESEARCH AND DEVELOPMENT CO. LTD. [IL/IL]; Weizmann Institute of Science, P.O. Box 95, 76100 Rehovot (IL).

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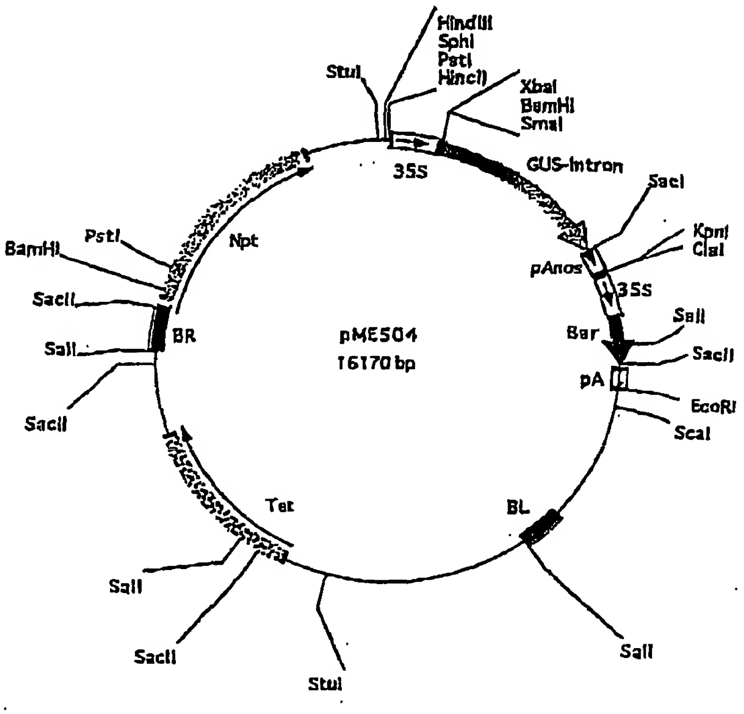
(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
With amended claims.

(54) Title: TRANSGENIC LEMNACEAE

(57) Abstract

The present invention concerns genetically stable transformed *Lemnaceae* plants and methods for their transformation by *Agrobacterium* cells. The present invention further concerns a method for regeneration of plants from *calli*, utilizing low sucrose media and products of interest produce from said plants. The present invention further concerns booster media for use in the above methods.



INTERNATI[illegible]L SEARCH REPORT

Ir. [illegible] Application No
PCT/IL 98/00487

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 C12N5/04 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FREY M. ET AL.: "Evidence for uptake of plasmid DNA into intact plants (Lemna perpusilla) proved by an E. coli transformation assay" ZEITSCHRIFT FÜR NATURFORSCHUNG, vol. 35c, 1980, pages 1104-1106, XP002067607 see the whole document ---	1,2
X	WO 89 12102 A (TEXAS A & M UNIVERSITY SYST) 14 December 1989 * see esp. p. 6 l. 25 ff. * ---	19,21,22
X	GB 2 211 204 A (OJI PAPER CO) 28 June 1989 see the whole document --- -/--	19,21,22

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search 6 January 1999	Date of mailing of the international search report 13/01/1999
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Kania, T

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INTERNATIO[illegible]L SEARCH REPORT

I[illegible]na[illegible]l Application No
PCT/IL 98/00487

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 15678 A (TEXAS A & M UNIVERSITY SYST ;ARNTZEN CHARLES J (US); MAY GREGORY D) 15 June 1995 see the whole document ---	19,27-32
X	EP 0 249 432 A (CALGENE INC) 16 December 1987 * see esp. p. 3 l. 41-55, p. 4 l. 1-9 * ---	37-41
X	MOON H. AND STOMP A.: "Effects of medium components and light on callus induction, growth,and frond regeneration in Lemna gibba (duckweed)" IN VITRO CELLULAR AND DEVELOPMENTAL BIOLOGY- PLANT, vol. 33, January 1997, pages 20-25, XP002067608 * see esp. p. 24 * ---	45,46, 48-51
X	WO 87 07299 A (CALGENE INC) 3 December 1987 * see esp. p. 13-15 * ---	47-53
A	VERNADE D. ET AL.: "Glycine betaine allows enhanced induction of the Agrobacterium tumefaciens vir genes by acetosyringone af low pH" JOURNAL OF BACTERIOLOGY, vol. 170, no. 12, December 1988, pages 5822-5829, XP002067609 see the whole document ---	28-32, 37-41
A	CHEMICAL ABSTRACTS, vol. 116, no. 12, 1991 Columbus, Ohio, US; abstract no. 123977, LIN T. ET AL.: "Effects of gamma-rays and caffeine on young inflorescence cultures of wheat" page 185; XP002067613 see abstract & HENONG XUEBAO 5 (1991),185-8, ---	31-33, 40-42
A	CHANG W.AND CHIU P.: "Regeneration of Lemna gibba G3 through callus culture" ZEITUNG FÜR PFLANZENPHYSIOLOGIE, vol. 89, 1978, pages 91-94, XP002067610 cited in the application see the whole document ---	1-57

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INTERNATI[illegible]L SEARCH REPORT

In[illegible]l Application No
PCT/IL 98/00487

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	CHANG W. AND HSING Y.: "Callus formation and regeneration of frond-like structures in Lemna perpusilla 6746 on a defined medium" PLANT SCIENCE LETTERS, vol. 13, 1978, pages 133-136, XP002067611 cited in the application see the whole document	1-57
A	TOBIN E. ET AL.: "Phytochrome regulation of transcription: biochemical and genetic approaches" NATO ASI SERIES, vol. H50, 1991, pages 167-179, XP002067612 * see p. 172-74 *	1-57
P,X	DE 196 29 402 A (VOESTE DIRK DR) 5 February 1998 see the whole document	1,2,6, 8-12,36, 54-57

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PATENT COOPERATION TR Y

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REC'D 28 FEB 2000
WIPO PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 114468.2 MM	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/IL98/00487	International filing date *(day/month/year)* 08/10/1998	Priority date *(day/month/year)* 10/10/1997
International Patent Classification (IPC) or national classification and IPC C12N15/82		
Applicant YEDA RESEARCH AND DEVELOPMENT CO. LTD. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 1 sheets.

3. This report contains indications relating to the following items:

   I ☒ Basis of the report
   II ☐ Priority
   III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   IV ☒ Lack of unity of invention
   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   VI ☐ Certain documents cited
   VII ☐ Certain defects in the international application
   VIII ☒ Certain observations on the international application

Date of submission of the demand 08/03/1999	Date of completion of this report 23.02.00
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Novak, S Telephone No. +49 89 2399 8930

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/IL98/00487

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-45	as originally filed

Claims, No.:

1-51	as originally filed
52-61	filed with the demand

Drawings, sheets:

1/1	as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

☐ restricted the claims.

☐ paid additional fees.

☐ paid additional fees under protest.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/IL98/00487

☐ neither restricted nor paid additional fees.

2. ☒ This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

☐ complied with.

☒ not complied with for the following reasons:

see separate sheet

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

☒ all parts.

☐ the parts relating to claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	3, 5 - 10, 13 - 18, 23 - 36, 40, 42 - 46, 52 - 61
	No:	Claims	1. 2. 4, 11, 12, 19 - 22, 37 - 39, 41, 47 - 51
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1 - 61
Industrial applicability (IA)	Yes:	Claims	1 - 61
	No:	Claims	

2. Citations and explanations

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

s e separate sheet

INTERNATIONAL PRELIMINARY EXAMINATION REPORT - SEPARATE SHEET International application No. PCT/IL98/00487

Reference is made to the following documents:

D1: FREY M. ET AL.: ZEITSCHRIFT FÜR NATURFORSCHUNG, vol. 35c, 1980, pages 1104-1106,
D2: GB-A-2 211 204 (OJI PAPER CO) 28 June 1989
D3: EP-A-0 249 432 (CALGENE INC) 9 June 1987
D4: WO 87 07299 A (CALGENE INC) 3 December 1987
D5: CHEMICAL ABSTRACTS, vol. 116, no. 12, 1991 Columbus, Ohio, US; abstract no. 123977, LIN T. ET AL.: page 185; XP002067613 & HENONG XUEBAO 5 (1991),185-8,

ad IV.

1. Lack of Unity (Rule 13 PCT)

1.1. The separate groups of invention are:
Claims 1 - 36, and 52 - 58: stably transformed *Lemnaceae* plants, respectively uses of these transformed plants, or methods for the transformation;
Claims 37 - 44: mediums for enhancing *Agrobacterium* cell virulence;
Claims 45 - 51, and 59 - 61: methods for maintaining morphogenetic *Lemnaceae* calli.
They are not so linked as to form a single general inventive concept (Rule 13.1 PCT) for the following reasons: these three groups of inventions do not have a common characteristic, essential technical feature, and as such are regarded as unrelated, separate inventions.

ad V.

2. Novelty (Article 33(2) PCT) and Inventive Step (Article 33(3) PCT)

2.1. Claims 1 to 18, and 54 to 57

Claim 1 is drawn to a genetically stable, transformed *Lemnaceae* plant and progeny thereof (see also item 3, Clarity).
D1 discloses evidence for uptake of plasmid DNA into *Lemna perpusilla* proved by

an *E.coli* transformation assay (see title). Following the incubation of *Lemna perpusilla* plants with the *E.coli* plasmids, uptake of the plasmids is shown by subsequent transformation of *E.coli* cells to tetracycline resistance after treatment with *Lemna* DNA from plasmid-incubated plants. In 7 out of 15 assays stable transformants are found (see abstract).
It follows from the teaching of D1 that novelty can not be acknowledged for the subject-matter of claim 1. The same applies to therefrom dependent claims 2, and 4, and independent claim 12 which deals with a method for the stable genetic transformation of *Lemnaceae* plants.

The additional subject-matter of dependent claims 3, and 5 to 10 is not known from any prior art document, but consists merely in the selection of a particular plant strain, respectively specific marker genes, or expressible genes. Such a selection can only be regarded as inventive, if the additional features present unexpected effects or properties. However, no such effects or properties are indicated in the application, and thus the claimed selection appears arbitrary. Therefore, claims 3, and 5 to 10 are not allowable according to Article 33(3) PCT.

For the same reasons the subject-matter of claims 54 to 57 is not regarded as involving an inventive step.

Inventive step will also not be acknowledged for the subject-matter of dependent claims 13 to 18. If the skilled person would like to target the plants meristematic tissue specifically, it appears obvious to select *A. tumefaciens* strains well known in the art capable of infecting specific tissues, or wounded tissues (claims 13 to 16). The same is true for the subject-matter of claims 17 and 18 which is regarded as technical measures falling within the scope of customary activities of the skilled person.

The subject-matter of claim 11 is defined in terms of a a product-by process. Please note that a chemical or biological product as such is not rendered novel by a new way of producing this product. It appears that a plurality of already known chemical or biological products fall within the scope of claim 11. Therefore, claim 11 is not allowable under Article 33(2) PCT.

2.2. Claims 19 to 36

Claim 19 deals with a method for the genetic transformation of a plant.
A process for the production of a plant transformant that also employs dividing the mass of shoot primordium into small sections and coculturing these sections with cells of *Agrobacterium tumefaciens* to transform the shoot primordia with a plasmid contained in the cells of *Agrobacterium tumefaciens* is described in D2 (see abstract). Furthermore it is mentioned that this process is applicable for various kinds of plants.
Consequently, the subject-matter of claim 19, and therefrom dependent claims 20 - 22 is not considered as novel.

A method for the stable transformation of a *Lemnaceae* plant according to claim 23 is regarded as a slight procedural modification of the method employed in D1, falling within the scope of customary activities of the skilled person. Accordingly, no inventive step will be considered.
For the same reasons the subject-matter of claim 25 does not seem to involve an inventive step.
Dependent claims 24, and 26 to 36 do not contain any features which, in combination with the features of any claim to which they refer, meet the requirements of the PCT in respect of inventive step, since said features are regarded as being standard for the skilled person.

2.3. Claims 37 to 44

A booster medium for enhancing *Agrobacterium* cell virulence is known from several prior art documents, e.g. D3.
D3 describes the use of a feeder plate to act as a nurse culture in order to enhance the efficiency of transformation. Convenient plant cell suspensions comprise plant cells from the family of *Solananceae*, particularly *Nicotiana*. The medium is buffered in the range of pH 5 - 6, and contains an appropriate amount of growth hormones.
It follows that novelty can not be acknowledged for the subject-matter of claims 37 to 39, and 41.

The subject-matter of claims 40, and 42 to 44 however is not disclosed in any prior art document, but inventive step is not allowable for these claims. D5 describes the beneficial effects of caffeine at certain concentrations in plant media for the rate of callus induction and callus differentiation. It appears therefore obvious from the combined teaching of these documents to arrive at the subject-matter of claims 40 and 42.
The same applies to the subject-matter of claims 43 and 44 which is regarded as minor technical difference that would fall within the scope of customary experimental activities of a person skilled in the art.

2.4. Claims 45 to 51, and 60 to 61

A method for the regeneration of plants from calli or for the production of highly regenerative calli under the conditions as set forth in claims 47 to 51 are known from D4. Therefore these claims do not fulfill the requirements of Article 33(2) PCT.

The subject-matter of claims 45, and 46, 60 and 61 which is drawn to *Lemnaceae* plants is regarded as novel. However, a method for maintaining morphogenetic *Brassica* calli is known from D4 (see claims 7, 11, and 13; see also page 14 and 15). In the light of this document it appears to be foreseeable that the same conditions would have similar effects on the calli of *Lemnaceae* plants.
It follows that claims 45, 46, 60, and 61 do not fulfill the criteria of Article 33(3) PCT.

ad VIII.

4. Clarity (Article 6 PCT)

The term "genetically stable, transformed" used in claim 1 is vague and unclear and leaves the reader in doubt as to the meaning of the technical feature to which it refers, thereby rendering the definition of the subject-matter of said claim unclear (Article 6 PCT).

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WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ^{4} : **C12N 15/00, 5/00, 5/02** **C12N 1/00, A01H 1/04**	**A1**	**(11) International Publication Number:** **WO 87/ 07299** **(43) International Publication Date:** 3 December 1987 (03.12.87)

(21) International Application Number: PCT/US87/01205

(22) International Filing Date: 26 May 1987 (26.05.87)

(31) Priority Application Number: 868,640

(32) Priority Date: 29 May 1986 (29.05.86)

(33) Priority Country: US

(71) Applicant: CALGENE, INC. [US/US]; 1920 Fifth Street, Suite F, Davis, CA 95616 (US).

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(74) Agent: ROWLAND, Bertram, I.; Leydig, Voit & Mayer, 350 Cambridge Avenue, Suite 200, Palo Alto, CA 94306 (US).

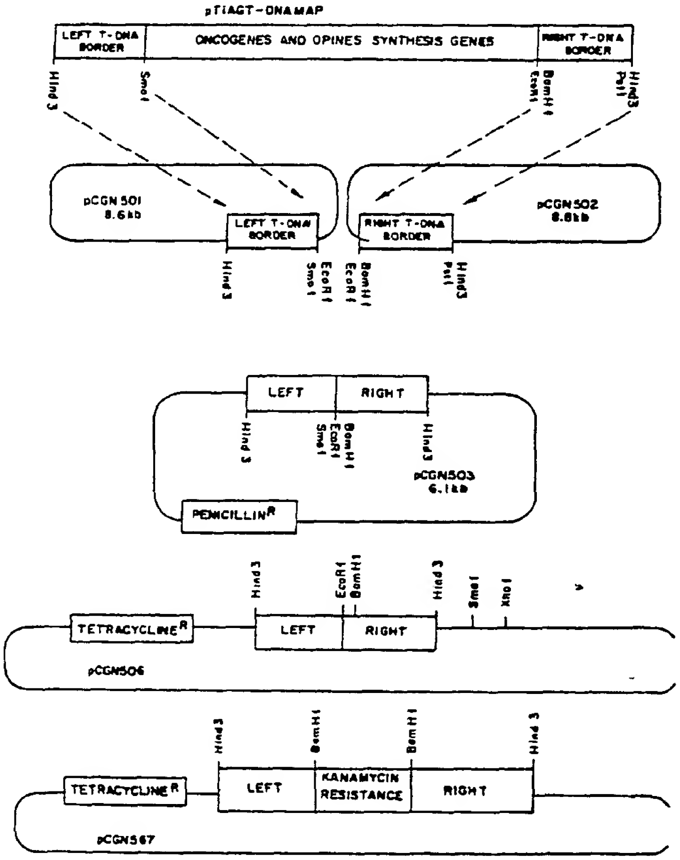
(81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), FI, FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), NO, SE, SE (European patent), SU.

Published
With international search report.

(54) Title: TRANSFORMATION AND FOREIGN GENE EXPRESSION IN *BRASSICA* SPECIES

(57) Abstract

Brassica species are produced by transformation of cell culture with foreign DNA followed by regeneration of plants from transformed cells. The cells and the plants produced thereby are capable of expressing the foreign gene. The *Brassica* species are transformed employing a manipulated *Agrobacterium* transformation system, followed by regeneration of the plant tissue into plants.



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SD	Sudan
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TRANSFORMATION AND FOREIGN GENE EXPRESSION IN BRASSICA SPECIES

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of Application Serial No. 868,640 filed March 29, 1986 and is herein incorporated by reference.

BACKGROUND OF THE INVENTION

Field of the Invention

This invention is related to a method for improving genotypes and associated phenotypes in Brassica species by means of Agrobacterium-based genetic transformation.

Background

Cruciferous species of the tribe Brassiceae are widely used as a source of protein, oil, condiments and chemical feedstocks. Significant effort has been expended to improve the quality of cultivated Brassica species by conventional plant breeding, and a number of major successes are recorded. The methods of conventional plant breeding have been limited, however, to the movement of genes and traits from members of the genus Brassica to others of the same genus and, in a few notable examples, "wide crosses" from other closely related genera. The development of a method for introducing foreign genes into Brassica species would greatly enhance the range of traits which could be imparted to Brassica oilseeds and vegetables.

In order to obtain a reliable system for useful gene introduction into Brassica species, a number of obstacles must be overcome. These include optimization of regenerability to whole plants of the target tissue, definition of the conditions (e.g., time, bac-

terial concentration, and media) for the co-cultivation of the Brassica cells and Agrobacterium cells, discovery of an Agrobacterium strain of suitable virulence with Brassica for gene transfer, identification of a suitable regulatory sequence (promoter) to ensure expression of the foreign gene in the transformed tissue, and expression of a selectable marker enabling identification of transformants.

Brief Description of Relevant Literature

Brassica species have been widely investigated for regenerability from tissue explants. Both Brassica napus and Brassica oleracea show shoot regeneration from a variety of tissues including hypocotyls (M.F. Dietert et al., Plant Science Letters (1982) 26:233-240), leaf callus (G.R. Stringham, Z. Pflanzenphysiol. (1979) 92:459-462), roots (P.A. Lazzeri and J.M. Dunwell, Annals of Botany (1984) 54:341-350), and leaf and stem protoplasts (L.C. Li and H.W. Kohlenbach, Plant Cell Reports (1982) 1:209-211; K. Glimelius, Physiol. Plant. (1984) 61:38-44). See also, the poster given by Radke, et al., Crucifer Genetics Workshop, Guelph, June 29, 1986.

The suitability of Agrobacterium as a vector for Brassica transformation is suggested by host range studies by DeCleene and DeLey (Botanical Rev. (1976) 42:386-466) demonstrating several species of Brassica (including napus, oleracea, nigra and campestris) to be susceptible to Agrobacterium. Recent studies by L.A. Holbrook and B.L. Miki (Plant Cell Reports (1985) 4:329-332) show some evidence for the expression of characteristic Agrobacterium genes in non-regenerable tumorous tissue.

The use of Agrobacterium tumefaciens for transforming plants using tissue explants is described in Horsch et al. (Science (1985) 228:1229-1231). See also, Herrera-Estrella et al. (Nature (1983) 303:209-

213), Fraley et al. (Proc. Natl. Acad. Sci. USA (1983) 80:4803-4807) and Bevan et al. (Nature (1983) 304:184-187). Use of the 35S promoter from cauliflower mosaic virus to direct expression of chimeric genes in plants has been reported (see C.K. Shewmaker et al., Virology (1985) 140:281-288 and R.C. Gardner et al., Plant Molecular Biology (1986) 6:221-228).

SUMMARY OF THE INVENTION

Transformed Brassica plants and tissues are provided which contain novel nucleotide constructions capable of stable expression. The transformation techniques employed are designed to optimize frequency of transformation, recovery of target tissue and regeneration of plants from the transformed tissue. Preferred techniques of the invention for obtaining the desired transformed Brassica plants include use of Agrobacterium tumefaciens strains (having virulence against Brassica), use of a medium during the selection process containing a less than normal carbon-source content (less than or equal to 2% sucrose or its equivalent in caloric value), use of efficient promoters, and use of hypocotyls as target tissues. The technique may make use of a feeder layer of tobacco suspension cells to assist with transformation frequency and recovery of the target tissue. Target tissue may include leaf or stem explants in addition to the hypocotyls discussed above.

Brief Description of the Figures

Fig. 1 is an autoradiography map created by polyacrylamide gel electrophoresis of cellular protein to demonstrate the presence of the neomycin phosphotransferase gene in transformed cells growing on selective media by showing the existence of ATP-mediated phosphorylation of kanamycin using γ-^{32}P-labelled ATP:

Lanes 1, 2, 3 and 4: *Brassica napus* cv Westar tissue transformed by A281x200 *Agrobacterium tumefaciens*.

Lanes 5 and 6: *Brassica napus* cv Westar tissue transformed by K12x200 *Agrobacterium tumefaciens*.

Lane 7: Negative control untransformed *Brassica* leaf tissue.

Lane 8: Bacterial neomycin phosphotransferase activity (positive control).

Fig. 2 is a graph showing sensitivity of transformed and untransformed *Brassica* tissue to kanamycin.

Fig. 3 is a pTiA6 T-DNA map and pathway to pTiK61.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Novel procedures and products are provided involving the introduction of novel nucleotide constructs into cells of *Brassica napus*, typically using leaf and hypocotyl explants, where the transformed cells of the plant express one or more genes present in the construct so as to provide at least one novel property for the plant, particularly a phenotypic property.

A number of process steps are involved in providing for efficient frequency of transformation, recovery of target tissue, and regeneration of plants from the transformed tissue. The initial stage is the selection of an *Agrobacterium tumefaciens* strain which provides for efficient transformation of *Brassica*. In the next stage, selection and regeneration media are employed with only a small amount of carbon-source materials (less than 2% sucrose or its equivalent in caloric value). The construct which is used should include a transcriptional initiation region which effi-

ciently functions in Brassica. The source of the plant cells for transformation is desirably from hypocotyls.

Agrobacterium Strains

Although any of the numerous strains of Agrobacterium tumefaciens capable of transferring genetic material to Brassica species can be used in combination with the other variations of the present invention, particularly improved transformation, recovery, and regeneration can be achieved by using Agrobacterium tumefaciens strains A281, EHA101, and K61, as well as other strains sharing common characteristics with these strains. These bacterial strains containing preferred plasmids (described later in detail) have been deposited with the American Type Culture Collection, Rockville, Maryland, and granted ATCC Deposit Accession No. A. tumefaciens A281 (pCGN200) ATCC No. 67121; A. tumefaciens (K61), ATCC No. ______________.

A number of Agrobacterium tumefaciens strains have been developed having different Ti-plasmids, e.g., agropine or nopaline specific. Both armed and disarmed plasmids are employed, that is, the armed plasmids contain oncogenic T-DNA that can be transferred to the plant cell and the disarmed plasmids contain no oncogenes that can be transferred to the plant cell. The strains include Agrobacterium tumefaciens strains A281, EHA101, and K61.

Agrobacterium A281, containing the Ti-plasmid from strain Bo542, has the following characteristics: biotype 1, agropine synthase and agropine catabolism positive, and 3-ketolactose positive. This armed strain was constructed by an in planta conjugation of wild-type Bo542 with strain A136, a C58 nopaline strain derivative cured of its Ti-plasmid and made resistant to rifampicin and nalidixic acid (E. Hood et al., Biotechnology (1984) 2:702-708; D. Sciaky et al., Plasmid (1978) 1:238-253). The virulence plasmid pTiBo542 is 249kD that contains an intact vir region and T-DNA region.

Strain EHA101 is described in Hood _et al._, _J. of Bacteriology_ (1986) _168_:1291-1301.

Strain K61 is derived from _Agrobacterium_ strain K12 by tripartite mating with pCGN567 containing the wide host range replication system pVK102 (Knauf and Nester, _Plasmid_ (1982) _8_:45-54) and the left and right T-DNA borders of pTi86.

Agrobacterium strain A348, (Garfinkel _et al._, _Cell_ (1981) _27_:143-153) containing pTiA6 was transformed into strain A114 or NT1 and the resulting strain selected for octopine catabolism on BTB media (Hooykaas _et al._, _J. Gen. Microbiol._ (1979) _110_:99-109) was named K12.

The _Agrobacterium_ to be employed as the transformation system is conveniently transformed with a wide-host-range plasmid that can shuttle DNA from _E. coli_ into _Agrobacterium_. This is achieved by having a P-1 incompatibility plasmid replicon, e.g., RK2, and a plasmid replicon capable of providing multicopies in _E. coli_, usually at least 5, preferably at least 10, and up to 200 copies in _E. coli_. The wide-host-range plasmid will be characterized by having at least one T-DNA border sequence, particularly the right border sequence, or conveniently having both border sequences separated in one direction by the various constructs intended to be integrated into the plant species genome. The _Agrobacterium_ strain may have either a disarmed Ti- or Ri-plasmid, as indicated above. The plasmid pCGN200 can be transformed into _A. tumefaciens_ and detected by kanamycin resistance. Plant cells may then be cocultivated with the _A. tumefaciens_ transformant, grown and selected for resistance to a biocide and expression of the desired gene(s) and can be monitored by Southern and Western blots, immunoassays, and the like. Of particular interest as markers are markers which impart biocide resistance to plant cells and plants, so that the transformed plant species can be efficiently selected.

The Transformation Process

The transformed plant cells may be cells in culture, cells present as a disorganized mass in a callus, cells organized as leaf explants, shoot cultures, seeds, fruits, leaves, roots, or cells organized as a whole plant. Hypocotyl segments are particularly preferred as target cells for forming the transformed plant cells as an enhanced transformation and recovery rate results from the use of hypocotyl segments. A hypocotyl is the part of the axis, or stem, below the cotyledons in the embryo of a plant.

The Agrobacterium strain will include on a plasmid, either the Ti-plasmid or the wide host range plasmid, a foreign construct, which foreign construct is destined to be transferred to the plant cell. As a result of such transfer, the foreign construct will normally be present in all or substantially all of the cells of the plant tissue after transformation and regeneration, but expression may be limited to particular cells or particular stages in the development of the plant. The foreign construct will include transcriptional and translational initiation and termination signals, with the initiation signals 5' to the gene of interest and the termination signals 3' to the gene of interest in the direction of transcription.

The transcriptional initiation region which includes the RNA polymerase binding site (promoter) may be native to the plant host or may be derived from an alternative source, where the region is functional in the Brassica host. A wide variety of transcriptional initiation regions may be employed, including those which are endogenous to Brassica or specific Brassica species, e.g. napus, or exogenous to Brassica, that is, comes from a cellular source other than a Brassica species cell. The sources of such transcriptional initiation regions may include other plant species, plant

viruses, and bacterial plasmids, such as the Ti- or Ri-plasmids, particularly the T-DNA genes which are functional in plant cells. The transcriptional initiation regions may be constitutive or regulatable. Regulatable genes may be regulatable by external signals, including physical signals such as light and heat, chemical signals, such as metabolites, or cell differentiation signals, such as root-specific, leaf-specific, seed-specific, etc., or stress-related signals, etc. A preferred promoter region is the 35S promoter from cauliflower mosaic virus. This promoter is well known but has not been used previously with Brassica.

The 3' termination region may be derived from the same gene as the transcriptional initiation region or a different gene. For example, where the gene of interest has a transcriptional termination region functional in a Brassica species, that region may be retained with the gene.

An expression cassette can be constructed which will include the transcriptional initiation region, the gene of interest under the transcriptional regulational control of the transcriptional initiation region, the initiation codon, the coding sequence of the gene (with or without introns), and the translational stop codons, and will be followed by the transcriptional termination region (which will include the terminator and may include a polyadenylation signal sequence and other sequences associated with transcriptional termination). The direction is 5'-3' in the direction of transcription. The cassette will usually be less than about 10kD, frequently less than about 6kD, usually being at least about 1kD, more usually being at least about 2kD.

The gene of interest may be derived from a chromosomal gene, cDNA, a synthetic gene, or combinations thereof. Where the expression product of the gene is to be located in other than the cytoplasm, the

gene will usually be constructed to include particular amino acid sequences which result in translocation of the product to a particular site, which may be an organelle (such as the chloroplast, mitochondrion or nucleus) or the cell plasma membrane, or the product may be secreted into the periplasmic space or into the external environment of the cell. Various secretory leaders, membrane integrator sequences, and translocation sequences (transit peptides) for directing the peptide expression product to a particular site are described in the literature. See, for example, Cashmore et al., Biotechnology (1985) 3:803-808; Wickner and Lodish, Science (1985) 230:400-407.

Genes of interest for use in Brassica species include a wide variety of phenotypic and non-phenotypic properties. Among the phenotypic properties are enzymes which provide for resistance to stress, such as dehydration resulting from heat and salinity, resistance to insects, herbicides, toxic metal or trace elements, or the like. Resistance may be as a result of a change in the target site, an enhancement of the amount of the target protein in the host cell, an increase in one or more enzymes involved with the biosynthetic pathway to a product which protects the host against the stress, or the like. Genes may be obtained from prokaryotes or eukaryotes, including but not limited to bacteria, fungi (e.g., yeast), viruses, plants, or mammals or may be synthesized in whole or in part. Illustrative genes include glyphosate resistant 3-enolpyruvylphosphoshikimate synthase gene, nitrilase, genes in the proline and glutamine biosynthetic pathway, and metallothioneins. Other genes of interest may be involved with regulation of growth, such as manipulations of source/sink (carbon partitioning) relations, hormonal regulation; resistance to herbicides, such as phenmedipham; production of male sterility; regulation of photosynthetic efficiency, such as altering the

efficiency of RuBP carboxylase; control of the quality of the plant taste or nutritional value; altering oil or protein profile, yield, or quality; or reduction of specific undesirable metabolites such as glucosinolates or extremely long chain fatty acids e.g., C_{22} fatty acids.

Instead of an expression cassette, one may have a transcription cassette, where the RNA sequence which is produced is complementary to an endogenous transcriptional product. The complementary or anti-sense sequence may be to an open reading frame, or a non-coding region, such as an intron or a 5'-non-coding leader sequence. In this way, the expression of various endogenous products may be modulated.

One or more cassettes may be involved, where the cassettes may be employed in tandem for the expression of independent genes which may express products independently of each other or may be regulated concurrently, where the products may act independently or in conjunction.

Where the expression cassette is to be transformed into plant cells by means of Agrobacterium, the cassette will be bordered by the right and optionally left T-DNA borders. These borders may be obtained from any Ti- or Ri-plasmid and may be joined to the expression cassette by conventional means. The expression cassette may be constructed so as to be directly transferred from a plasmid other than a Ti- or Ri-plasmid or may become integrated into the T-DNA region of a Ti- or Ri-plasmid through homologous recombination. Thus, the expression cassette could have DNA sequences at one or both borders of the expression cassette homologous with sequences present in the T-DNA region of the Ti- or Ri-plasmid.

The expression cassette will normally be carried on a vector having at least one replication system. For convenience, it is common to have a replica-

tion system functional in *E. coli*, such as *ColE1*, pSC101, pACYC184, or the like. In this manner, at each stage after each manipulation the resulting construct may be cloned and sequenced, and the correctness of the manipulation can be determined. In addition to or in place of the *E. coli* replication system, a broad host range replication system may be employed, such as the replication systems of the P-1 incompatibility plasmids; e.g., pRK290. These plasmids are particularly effective with armed and disarmed Ti-plasmids for transfer of T-DNA to the plant species host.

In addition to the replication system, there will frequently be at least one marker present, which may be useful in one or more hosts, or different markers for individual hosts. That is, one marker may be employed for selection in a prokaryotic host, while another marker may be employed for selection in a eukaryotic host, particularly the plant species host. The markers may be protection against a biocide, such as antibiotics, toxins, heavy metals, or the like, or may function by complementation, imparting prototrophy to an auxotrophic host. Various genes which may be employed include neomycin phosphotransferase (NPTII; also known as APHII), hygromycin phosphotransferase (HPT), chloramphenicol acetyltransferase (CAT), nitrilase, and gentamicin resistance genes. For plant host selection, markers of particular interest include NPTII, providing kanamycin resistance or G418 resistance; HPT, providing hygromycin resistance; CAT, providing chloramphenicol resistance; mutated *AroA* gene, providing glyphosate resistance; etc.

The various fragments comprising the various constructs, expression cassettes, markers, and the like may be introduced consecutively by restriction enzyme cleavage of an appropriate replication system and insertion of the particular construct or fragment into the available site. After ligation and cloning the

vector may be isolated for further manipulation. All of these techniques are amply exemplified in the literature and find particular exemplification in Maniatis _et al._, _Molecular Cloning: A Laboratory Manual_, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982.

Once the vector is complete, the construct may now be introduced into plant cells. Techniques for transforming plant cells include microinjection, direct DNA uptake using polyethylene glycol, electroporation, viral infection, and transformation with _Agrobacterium_. In accordance with the subject invention, a functional method has been developed for transformation of _Brassica_ cells employing _Agrobacterium_. This technique provides a methodology for the transformation of plant species with foreign genes in an efficient manner, so as to provide a rapid technique for transforming plant cells and regeneration of plants in an efficient reproducible manner.

Target Tissues

Although the prior art teaches that _Brassica_ can be regenerated from numerous plant tissues, hypocotyls have been found to provide the greatest efficiency of transformation. Other plant parts such as leaf explants, may be used in conjunction with the subject selection and regeneration medium. However, use of hypocotyl tissue represents a preferred embodiment of the present invention.

Sterile seeds are preferably employed as a source for _Brassica_ hypocotyls. Surface sterilized leaf pieces from 3-week-old plants or sterile grown hypocotyls of _Brassica napus_ cv Westar both regenerate readily. The cut surfaces of these explants provides an ideal _Agrobacterium_ target.

Any _Brassica_ species can be used in the practice of the present invention such as _B. napus_ (rape seed and rutabaga), _B. oleraceae_ (cabbage, broccoli,

brussel sprouts and other oleracea varieties), B. juncea (indian mustard), B. campestris (turnip rape), and the like.

Use of Feeder Cells During Transformation

Feeder cells can be used in the transformation process. The cells of the feeder plates act as a nurse culture for the Brassica explant as well as enhancing the efficiency of the transformation rate. In general tobacco feeder cells are used for ease of manipulation. Other feeder cells could be employed, particularly Brassica feeder cells in the form of a fine suspension.

The feeder plates are prepared by employing a plant suspension culture (e.g., Nicotiana cells grown in Murashige minimal organic medium (Flow Lab.) supplemented with 0.2 mg/l 2,4-dichlorophenoxyacetic acid and 0.1 mg/l kinetin) on a soft agar medium, generally having from about 0.5 to 1% agar and containing an appropriate growth medium, such as Gamborg, Miller and Ojima salts (B5 salts) ref. Gamborg et al., Exp. Cell. Res. (1968) 50:151-158, a carbon source, e.g., sucrose (3%), and appropriate amounts of growth substances, i.e., auxins, such as 2,4-dichlorophenoxyacetic acid (2,4-D), kinetin and vitamins (such as thiamine), with the medium appropriately buffered in the range from 5 to 6, preferably about 5.5. 2,4-D and kinetin concentrations are 1 mg/l. The final concentration of the vitamins and supplements is as follows: Inositol (100 mg/l), Nicotinic acid (1mg/l), Pyridoxine HCl (1 mg/l), Thiamine HCl (10 mg/l). Desirably, the feeder plates are prepared prior to being used, usually 24-48 hours before being used.

The feeder plates are covered with a porous cover to prevent the feeder cells from coming into contact with the Brassica leaf or shoot explants. This porous cover allows the explants to be bathed in conditioned medium. This can be readily achieved employing

a sterile filter paper disk. The explants are then allowed to preincubate, followed by transfer to a broth culture of the Agrobacterium strain containing the construction for integration and having the genetic capability for transfer of the construct into the plant cells. Generally, the number of bacteria is from about 10^7 to 10^9/ml (final concentration). The contact time with the bacteria in the bacterial broth culture, e.g., MG/L (same as LBMG; see Garfinkel *et al*., *J. Bacteriol.* (1980) **144**:732-743), is preferably about 30 minutes to 1 hour, although longer or shorter times may be used. The explants are then transferred from the bacterial broth, excess surface liquid is removed, and the sections are returned to the feeder plates. Bacterial co-cultivation on the feeder plates is for at least 12 hours and not more than 3 days, averaging 1-2 days.

Selection and Regeneration Procedures

It has been found that use of a low-carbon-source medium during the regeneration procedure following co-cultivation of the *Brassica* cells with the transforming bacteria results in enhanced recovery and regeneration. Use of a low-carbon-source medium is believed to operate by forcing the cultured tissues to become dependent on other sources of energy, possibly photosynthesis. A low-carbon-source medium is one that contains less than 2% by weight sucrose or the equivalent of a 2% by weight sucrose solution in caloric value. Other carbon sources (e.g., mono- or di-saccharides) may give a similar effect provided that they provide the same caloric value. A typical salt and vitamin mixture is employed in conjunction with cytokinins for regeneration media.

After the 1-2 day co-cultivation with the bacteria described above, the explants are typically transferred to a *Brassica* callus media (which preferably contains B5 salts and vitamins, 1mg/ 2,4-D and

kinetin, 3% sucrose). Cytokinins may be excluded and their absence enhances regeneration frequency. The callus forming medium contains a bacteriocide, e.g., carbenicillin (500 mg/L) and a selective agent may be applied. For example, with the kanamycin resistance gene (neomycin phosphotransferase, NPTII) as the selective marker, kanamycin at a concentration of from about 10 to 200 mg/l may be included in the medium. Typical concentrations for selection are 10-50 mg/l although some transformants may be resistant to 200 mg/l kanamycin. The tissue is grown upon this medium for a period of 1 to 3 weeks, preferably about 7 days.

After this time the callusing explants are transferred to Brassica regeneration medium. This medium contains Gamborg, Miller and Ojima B5 salts and vitamins as described below, 1% sucrose, 3-benzyladenine (3 mg/l), zeatin (1 mg/l) 0.6% purified agar (Phytagar, Gibco), and carbenicillin at 500 mg/l. At this stage a selective agent may be applied. Shoot formation begins in about 3-6 weeks depending on treatment and co-cultivation conditions. Kanamycin-resistant callus, which is also potentially regenerable, grows in a similar time. Both regenerants and transformed callus are removed and regularly (every other week) transferred to fresh B5 medium containing the other components described immediately above. Failure to perform regular transfers results in loss of transformants and depression of apparent transformation rate.

The Brassica transformation and regeneration system described above has been found to be rapid and efficient. A sufficient percentage of the co-cultivated explants are transformed in order to provide an economic system for transforming Brassica.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

E. coli strain MM294 (Hanahan, *J. Mol. Biol.* (1983) *116*:557-580) was used as the host for binary vectors containing the pRK290 type replicon. Strain K12 was generated by transforming pTiA6 into strain A114 (NT1) (Nester and Kosuge, *Ann. Rev. Microbiol.* (1981) *35*:531; Hoekema *et al.*, *Nature* (1983) *303*:179). *Agrobacterium* strain A281 was generated by conjugating wild-type Bo542, with strain A136 (D. Sciaky, *et al.*, *Plasmid* (1978) *1*:238-253). *Agrobacterium* strain EHA101 is described by Hood *et al*. *J. of Bacteriology* (1986) *168*: 1291-130)

Levels of antibiotics used with *E. coli* in mg/l were 30 for kanamycin, 50 for chloramphenicol, 300 for penicillin, 10 for tetracycline, and 20 for gentamicin. Unless otherwise indicated, levels of antibiotics used with *Agrobacterium* in mg/l were 100 for kanamycin or gentamicin and 50 for carbenicillin or chloramphenicol.

Laboratory Procedures

Restriction enzymes and T4 ligase were obtained from commercial sources and used according to manufacturers' recommendations. Standard methods of cloning and molecular analysis were performed as described in Maniatis *et al.*, *supra*.

Designations of media used in these procedures are as follows:

B5 medium means that described by Gamborg, Miller and Ojima, *Exp. Cell Research* (1968) *50*:151-158.

B5 Salts are:	mg/l
Ammonium sulfate	134.0
Boric acid	3.0
Calcium chloride	150.0
Cobaltous chloride	0.025
Cupric sulfate	0.025
Ferrous sulfate	27.8
Magnesium sulfate	250.0
Manganese sulfate	10.0
Potassium iodide	0.75
Potassium nitrate	2500.0
Sodium ethylenediamine tetraacetate	37.3
Sodium molybdate	0.25
Sodium dihydrogen phosphate	150.0
Zinc sulfate	2.0

Unless otherwise stated B5 medium contains 3% sucrose weight/volume.

B5 vitamins and supplements are:	mg/l
myo-Inositol	100.0
Nicotinic acid	1.0
Pyridoxine HCl	1.0
Thiamine HCl	10.0

The designation B5 0/1/1 means B5 medium, 3% sucrose, 1 mg/l 2,4-D, 1 mg/l kinetin and the designation B5 0/1/0 is the same except the kinetin is not included. The growth substance concentrations are denoted by the / (slash) notation, with the concentration of 3 growth substances in mg/l being denoted. In order these are indole-3-acetic acid/2,4-D/kinetin.

The designation B5BZ 1% means B5 salts, vitamins and supplements plus 1% w/v sucrose, 3 mg/l benzyl adenine and 1 mg/l zeatin. B5BZ 1% is used here as regeneration and selection medium for kanamycin resistance of *Brassica* tissue.

Example I

Construction of pCGN587

The BglII-SmaI fragment of Tn5 containing the entire structural gene for APHII (Jorgensen et al., Mol. Gen. (1979) 177:65) was cloned into pUC8 (Vieira and Messing, Gene (1982) 19:259), converting the fragment into a HindIII-EcoRI fragment, since there is an EcoRI site immediately adjacent to the SmaI site. The PstI-EcoRI fragment containing the 3' portion of the APHII gene was then combined with an EcoRI-BamHI-SalI-PstI linker into the EcoRI site of pUC7 (pCGN546W). Since this construct does not confer kanamycin resistance, kanamycin resistance was obtained by inserting the BglII-PstI fragment of the APHII gene into the BamHI-PstI site (pCGN546X). This procedure reassembles the APHII gene, so that EcoRI sites flank the gene. An ATG codon was upstream from and out of reading frame with the ATG initiation codon of APHII. The undesired ATG was avoided by inserting a SauIIIA-PstI fragment from the 5'-end of APHII, which fragment lacks the superfluous ATG, into the BamHI-PstI site of pCGN546W to provide plasmid pCGN550.

The EcoRI fragment containing the APHII gene (1ATG) was then cloned into the unique EcoRI site of pCGN451, which contains an octopine synthase cassette for expression to provide pCGN552(1ATG).

Plasmid pCGN451 includes an octopine cassette which contains about 1556bp of the of the 5' non-coding region fused via an EcoRI linker to the 3' non-coding region of the octopine synthase gene of pTiA6. The pTi coordinates are 11,207 to 12,823 for the 3' region and 13,643 to 15,208 for the 5' region as defined by Barker et al., Plant Mol. Biol. (1983) 2:325.

The 5' fragment was obtained as follows. A small subcloned fragment containing the 5' end of the coding region, as a BamHI-EcoRI fragment was cloned in

pBR322 as plasmid pCGN407. The BamHI-EcoRI fragment has an XmnI site in the coding region, while pBR322 has two XmnI sites. pCGN407 was digested with XmnI, resected with Bal31 nuclease and EcoRI linkers added to the fragments. After EcoRI and BamHI digestion, the fragments were size fractionated, the fractions cloned and sequenced. In one case, the entire coding region and 10bp of the 5' non-translated sequences had been removed leaving the 5' non-transcribed region, the mRNA cap site and 16bp of the 5' non-translated region (to a BamHI site) intact. This small fragment was obtained by size fractionation on a 7% acrylamide gel and fragments approximately 130bp long eluted. This size fractionated DNA was ligated into M13mp9 and several clones sequenced and the sequence compared to the known sequence of the octopine synthase gene. The M13 construct was designated pI4, which plasmid was digested with BamHI and EcoRI to provide the small fragment which was ligated to a XhoI to BamHI fragment containing upstream 5' sequences from pTiA6 (Garfinkel and Nester, J. Bacteriol. (1980) 144:732) and to an EcoRI to XhoI fragment containing the 3' sequences. The resulting XhoI fragment was cloned into the XhoI site of a pUC8 derivative, designated pCGN426. This plasmid differs from pUC8 by having the sole EcoRI site filled in with DNA polymerase I, and having lost the PstI and HindIII site by nuclease contamination of HincII restriction endonuclease, when a XhoI linker was inserted into the unique HincII site of pUC8. The resulting plasmid pCGN451 has a single EcoRI site for the insertion of protein coding sequences between the 5' non-coding region (which contains 1,550bp of 5' non-transcribed sequences including the right border of the T-DNA, the mRNA cap site and 16bp of 5' non-translated sequence) and the 3' region (which contains 267bp of the coding region, the stop codon, 196bp of 3' non-translated DNA, the polyA site and 1,153bp of 3' non-transcribed sequence).

The resulting plasmid pCGN451 having the ocs 5' and the ocs 3' in the proper orientation was digested with EcoRI and the EcoRI fragment from pCGN451 containing the intact kanamycin resistance gene inserted into the EcoRI site to provide pCGN552 having the kanamycin resistance gene in the proper orientation.

This ocs/KAN gene was used to provide a selectable marker for the trans-type binary vector pCGN587.

The 5' portion of the engineered octopine synthase promoter cassette consists of TiA6 DNA from the XhoI fragment at bp 15208-13644 (Barker's numbering as in Barker *et al.* *supra*), which also contains the T-DNA boundary sequence (border) implicated in T-DNA transfer. In the plasmid pCGN587, the ocs/KAN gene from pCGN552 provides a selectable marker as well as the right border. The left boundary region was recloned from the HindIII-EcoI fragment as a KpnI-EcoRI fragment in pCGN565 to provide pCGN580. pCGN565 is a cloning vector based on pUC8-Cm, but containing pUC18 linkers. pCGN580 was linearized with BamHI and used to replace the smaller BglII fragment of pVCK102 (Knauf and Nester, *Plasmid* (1982) 8:45), creating pCGN585. By replacing the smaller SalI fragment of pCGN585 with the XhoI fragment from pCGN552 containing the ocs/KAN gene, pCGN587 was obtained.

Construction of pCGN200

To construct pCGN200, a plasmid containing a full-length promoter from CaMV (35S) and kanamycin gene as a selectable marker, pCGN167 was recombined into a binary vector, pCGN587.

To construct pCGN167, the AluI fragment of CaMV (bp 7144-7735) (R. Gardner *et al.*, *Nucl. Acid Res.* (1981) 9:2871-2888) was obtained by digestion with AluI and cloned into the HincII site of M13mp7 (Vieira, *et al.*, *Gene* (1982) 19:259) to create C614. An EcoRI

digest of C614 produced the EcoRI fragment from C614 containing the 35S promoter which was cloned into the EcoRI site of pUC8 (Vieira et al., Gene (1982) 19:259) to produce pCGN146.

To trim the promoter region, the BglII site (bp 7670) was treated with BglII and Bal31 and subsequently a BglII linker was attached to the Bal31 treated DNA to produce pCGN147.

pCGN148a containing a promoter region, selectable marker (KAN with 2 ATG's) and 3' region, was prepared by digesting pCGN528 (see below) with BglII and inserting the BamHI-BglII promoter fragment from pCGN147. This fragment was cloned into the BglII site of pCGN528 so that the BglII site was proximal to the kanamycin gene of pCGN528.

The shuttle vector used for this construct, pCGN528, was made as follows. pCGN525 was made by digesting a plasmid containing Tn5 which harbors a kanamycin gene (Jorgenson et al., Mol. Gen. (1979) 177:65) with HindIII-BamHI and inserting the HindIII-BamHI fragment containing the kanamycin gene into the HindIII-BamHI sites in the tetracycline gene of pACYC184 (Chang & Cohen, J. Bacteriol. (1978) 134:1141-1156). pCGN526 was made by inserting the BamHI fragment 19 of pTiA6 (Thomashaw et al., Cell (1980) 19:729-739) into the BamHI site of pCGN525. pCGN528 was obtained by deleting the small XhoI fragment from pCGN526 by digesting with XhoI and religating.

pCGN149a was made by cloning the BamHI-kanamycin gene fragment from pMB9KanXXI into the BamHI site of pCGN148a.

pMB9KanXXI is a pUC4K variant (Vieira & Messing, Gene (1982) 19:259-268) which has the XhoI site missing but contains a functional kanamycin gene from Tn903 to allow for efficient selection in Agrobacterium.

pCGN149a was digested with BglII and SphI. This small BglII-SphI fragment of pCGN149a was replaced with the BamHI-SphI fragment from MI (see below) isolated by digestion with BamHI and SphI. This produces pCGN167, a construct containing a full length CaMV promoter, 1ATG-kanamycin gene, a 3' end and the bacterial Tn903-type kanamycin gene. MI is an EcoRI fragment from pCGN546X (see construction of pCGN587) and was cloned into the EcoRI cloning site of M13mp9 in such a way that the PstI site in the 1ATG-kanamycin gene was proximal to the polylinker region of M13mp9.

pCGN200 was made by transforming an *E. coli* strain C2110 (polA1) containing the binary vector pCGN587 with pCGN167. pCGN167 recombined *in vivo* to make pCGN200. There are two regions of direct DNA homology by which recombination could have occurred. In this case recombination took place between the pUC origin of replication regions carried by pCGN167 and pCGN587. Recombinants were selected by kanamycin resistance (deFromard *et al.*, *Biotechnology*, May 1983, pp. 262-267).

pCGN200 was introduced into *Agrobacterium tumefaciens* A281 and K12 by mating. Bacterial matings were performed using two *E. coli* strains and one *Agrobacterium* strain. One *E. coli* strain (MM294) harbored pRK2073 which provided mobilization functions, and the other strain (C2110) carried the plasmid with a kanamycin resistance marker to be transferred into *Agrobacterium*. The two *E. coli* strains were grown overnight at 37°C with shaking in LB broth. The *Agrobacterium* strain was grown overnight at 28°C in MG/L broth. Fifty microliters of each of the three strains were mixed on a nitrocellulose filter and placed on an MG/L plate. The plate was incubated at 28°C for 3 days. The mixture was then streaked onto an AB minimal medium (D.M. Glover, *DNA Cloning Volume II* (1985) p. 78) supplemented with 100 µg/ml kanamycin and

100 µg/ml streptomycin and incubated at 28°C for two days. Streptomycin was included to kill the two *E. coli* strains. Single colonies were picked and purified by two more streakings on the above medium.

Construction of strain K61

The Ti plasmid pTiA6 was isolated from *Agrobacterium* strain A348 (Garfinkel *et al.*, *Cell* (1981) *27*:143-153) and used to transform *Agrobacterium* strain A114 (also designated NT1) (Currier and Nester, *J. Bacteriol.* (1976) *126*:157-165). Octopine catabolism was selected for on BTB media (Hooykaas *et al.*, *J. Gen. Microbiol.* (1979) *110*:99-109). This strain was named K12.

The left T-DNA border region of pTiA6 (bp 602 to 2212 by the numbering system of Barker *et al.*, *supra*) was cloned as a *Hind*III to *Sma*I fragment in the phage vector M13mp9 (pCGN501). The M13mp9 linker architecture provides the fragment as a *Hind*III to *Eco*RI fragment. The right T-DNA border region of pTiA6 (bp 13362 to 15208, Barker *et al.*, *supra*) was subcloned as a *Eco*RI to *Xho*I fragment into M13mp9 cut with enzymes *Eco*RI and *Sal*I (pCGN502). This piece could then be excised as a *Eco*RI to *Hind*III fragment. The plasmids pCGN501 and pCGN502 were digested with *Hind*III and *Eco*RI and ligated to pUC8 DNA previously cut with just *Hind*III. Selection of white penicillin resistant colonies yielded an isolate containing the pUC8 vector with a 3.5 kbp *Hind*III fragment containing the left and right T-DNA borders of pTiA6 in the natural orientation (pCGN503). This 3.5 kpb *Hind*III fragment containing T-DNA borders was then transferred into the *Hind*III site of pVK102 (pCGN506). The wide host range cloning vector pVK102 (also designated pVCK102) has been described (Knauf and Nester, *Plasmid* (1982) *8*:45-54). The orientation of the border fragment in pCGN506 relative to the pVK102 vector is the left T-DNA border

region proximal to the tetracycline resistance locus. pCGN506 has a unique EcoRI site and a unique BamHI site in between the left and right border regions, i.e., such that any inserts would be oriented in the natural orientation. The plasmid pCGN567 was constructed by ligating the BamHI fragment of pUC4K bearing a kanamycin resistance determinant into the BamHI site of pCGN506. Thus, pCGN567 codes for both tetracycline and kanamycin resistance.

The plasmid pCGN567 was mated into *Agrobacterium* strain K12 by the tripartite method (Ditta *et al.*, *Proc. Natl. Acad. Sci. USA* (1981) 77:7347-7351) using kanamycin selection for transconjugant *Agrobacterium* on minimal media. *E. coli* bearing the plasmid pPH1J1 (Garfinkel *et al*, *Cell* (1981) 27:143-153) was then mated with strain K12 (pCGN567) and transconjugant *Agrobacterium* were selected on minimal media containing both kanamycin and gentamicin. Since pPH1J1 and pCGN567 are incompatible plasmids, it was expected that a double recombination event between two direct regions of homology with the Ti-plasmid would result in the exchange of the kanamycin resistance locus for all of the oncogenic gene loci between the border regions (for explanation of method, see Garfinkel *et al.*, *Cell* (1981) 27:143-153,). However, this did not occur. Kanamycin and gentamicin resistant *Agrobacterium* resulting from the introduction of pPH1JI into K12 (pCGN567) grew very slowly suggesting both pCGN567 and pPH1JI were present in an unstable situation resulting in the apparent slow growth of a colony as individual bacteria tended to shed one of the two plasmids due to incompatibility problems. By alternating growth on kanamycin-containing media and gentamicin-containing liquid media in overnight cultures, an isolate with the expected growth rates was identified. Plasmid DNA was isolated from that strain and used to transform strain A114, selecting for kanamycin resistance and tooth-

picking for gentamicin sensitivity. The resulting strain of *Agrobacterium*, K61, therefore lacked pPH1JI. Restriction enzyme analysis of the plasmid in K61 (pTiK61) using *Sma*I, *Hpa*I, *Eco*RI, and *Sal*I revealed a Ti-plasmid lacking sequence corresponding to kbp 173.30 to 181 and 0.0 to 53.35 of pTiA6 (using the numbering system of Knauf and Nester, *Plasmid* (1982) *8*:45-54). This explained the inability of strain K61 to utilize octopine, a trait normally encoded by pTiA6. Thus, pTiK61 represents a spontaneous deletion of pTiA6 in which the left border region is still present but the oncogenes, right T-DNA border, the T_R-DNA region and other loci including octopine catabolism are deleted. Regions encoding the *vir* region of pTiA6, namely coordinates 111 through 168 (using the numbering system of Knauf and Nester, *supra*) are intact in pTiK61 which represents a disarmed Ti-plasmid lacking oncogenes or functional T-DNA but containing *vir* genes necessary to accomplish T-DNA transfer from binary T-DNA vectors.

Construction of pCGN767

A genomic library was constructed in the λ vector EMBL4 (Fischauf *et al.*, *J. Mol. Biol.* (1983) *170*:827-842) from *B. napus* DNA digested partially with *Sau*IIIA. Two unique napin genomic clones, designated λBnNa and λBnNb, were isolated when 4 x 10^5 recombinant phage were screened by plaque hybridization with a nick-translated pN1 napin cDNA probe (Crouch *et al.*, *J. Mol. Appl. Gen.* (1983) *2*:273-283).

The napin genomic clones were analyzed by restriction nuclease mapping and Southern blot hybridizations. Each phage contains just one napin gene, and only the napin gene region hybridizes to cDNA made from embryo RNA. The 3.3kb *Eco*RI fragment containing the λBnNa napin gene was subcloned in pUC8 (Veiera and Messing, 1982) and designated pgNa.

An approximate 320 bp *SalI* fragment was cloned into the *XhoI* site of pgNa to create pCGN714 placing a bacterial DNA sequence as a "tag" after the stop codon of the napin coding region in pgNa. In this case, the bacterial DNA sequence consisted of a *SalI* restriction fragment containing the coding region of a dihydrofolate reductase (DHFR) gene. The *EcoRI* fragment in pgNa containing the napin gene with 300 bp of promoter and approximately 2100 bp following the napin coding region is about 3.6 kb in pCGN714.

The *HindIII*-*EcoRI* set of linkers in pUC18 (Yanisch-Perron *et al.*, *Gene* (1985) *33*:103) were transferred into pUC12Cm (Keith J. Buckley, Ph.D. Thesis, USCD, 1985) to create pCGN565 which is basically a pUC replicon linked to chloramphenicol resistance and the blue-white cloning system of pUC12. The *EcoRI* fragment of pCGN714 containing the tagged napin gene was transferred to the *EcoRI* site of pCGN565 to get pCGN723 which codes for chloramphenicol resistance rather than the penicillin resistance of pCGN714. The *HindIII*-*EcoRI* linkers of pUC8Cm (Keith J. Buckley, Ph.D., Thesis, UCSD, 1985) were transferred into *HindIII*-*EcoRI* cut pEMBL19 (Dente *et al.* *Nucl. Acids. Res.* (1983) *11*:1645) making pCGN730a. Unlike the parent pEMBL19, pCGN730a lacks any *SstI* sites. The *EcoRI* fragment of pCGN723 containing the tagged napin gene was transferred to the *EcoRI* site of pCGN730a. The *SstI* site in the napin gene was then unique in the resulting plasmid, pCGN735.

Two 27mer oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer machine. #A consisted of the sequence:

C-C-T-G-A-T-G-A-T-G-A-T-G-A-T-G-A-T-G-C-T-G-C-A-G-C-T

in the order of 5' to 3'. #B consisted of the sequence:

G-C-A-G-C-A-T-C-A-T-C-A-T-C-A-T-C-A-T-C-A-G-G-A-G-C-T-

These two oligonucleotides are partially complementary so that by annealing, they leave 3' sticky ends compatible with cloning in SstI sites. In the desired orientation, a single insert adds coding for an additional 9 amino acids, 5 of which are methionine residues. The synthetic dsDNA created by annealing oligonucleotides #A and #B was cloned into the SstI site of pCGN735. Since the oligonucleotides were not phosphorylated, the insertion of only one element was likely even though the insert was in excess to pCGN735 SstI ends. Restriction enzyme analysis of that plasmid, pCGN757, indicated that an insert was present (this and the orientation were later verified by DNA sequencing). The EcoRI fragment of pCGN757 was transferred to pCGN565 so that plasmid pCGN757c, coded for chloramphenicol resistance rather than the penicillin resistance of pCGN757,

pCGN549 was made by cloning the EcoRI (coding for gentamicin resistance) of pPH1J1 (Hirsch and Beringer, Plasmid (1984) 12:139) into EcoRI and PstI cut pUC9. pCGN594 was made by cutting pCGN587 with HindIII and BglII and combining with pCGN549 cut with HindIII and BamHI. This replaced the pUC replicon and chloramphenicol marker of pCGN587 with a bacterial gentamicin marker. pCGN739 was made by replacing the HindIII-BamHI fragment of pCGN594 with the HindIII-BamHI linkers of pUC18. This effectively replaced the eukaryotic selectable marker of pCGN594 with a series of unique cloning sites for insertion of other types of selectable markers. pCGN763 was made by the transfer of the HindIII-BamHI fragment of pCGN976 (obtained by insertion of a HindIII-BamHI fragment from pCGN167 into

HindIII-BamHI digested pUC19 to introduce the 35S promoter, kanamycin resistance and tml 3' region) into HindIII-BamHI cut pCGN739. pCGN757c was linearized with HindIII and cloned into the HindIII site of pCGN763 to create the binary vector pCGN767 which includes a bacterial gentamicin resistance marker, a chimeric eukaryotic kanamycin resistance gene (with CaMV 35S promoter and pTiA6 T-DNA tml transcription termination signals), and the tagged, engineered napin gene between pTiA6 T-DNA borders.

Example II

Brassica Transformation

Explants from soil-grown seedlings of Brassica napus cv Westar (Agriculture Canada, Saskatoon, Canada) were used as primary target material. Plants were grown 3-4 weeks in a 16-8 hour light-dark cycle 220 $\mu Em^{-2}S^{-1}$ at 24°C. Partially expanded secondary leaves were excised, surface sterilized for 15 min in 1% sodium hypochlorite, and washed four times with sterile water.

Leaf discs 4 mm in diameter were cut from the sterile leaves using a cork borer. These discs were pre-incubated for 24 hours at 24°C in darkness on a B5 medium (KC Biologicals) containing 1 mg/l 2,4-D and 1 mg/l kinetin solidified using 0.6% purified agar (Phytagar) (B5 0/1/1).

Agrobacterium tumefaciens (strain A281x200) was prepared overnight in MG/L broth by incubating a single colony of Agrobacterium tumefaciens. Bacteria were harvested at time periods of 16-36 hours. Dilutions of bacteria to concentrations of 10^6-10^7 bacteria per ml were prepared in B5 0/1/1 liquid medium. Leaf explants were inoculated with bacteria by dipping into the Agrobacterium suspension and then lightly blotting them on sterile paper towels. Inoculated leaf discs were then transferred to Petri plates of B5 0/1/1

medium with 0.6% Difco Phytagar, at a density of 20 discs per plate.

The co-incubation of bacteria and leaf discs took place for periods from 12 to 48 hours. After this co-culture step, the discs were washed in liquid B5 0/1/1 medium and transferred to Petri plates containing B5 0/1/1 and 500 mg/l carbenicillin, 0.6% Difco Phytagar. These explants were cultured in light ($50\mu Em^{-2}s^{-1}$) on this medium for 7-10 days until callus formation was evident. At this time the explants were transferred to a second medium optimized for regeneration in Brassica napus cv Westar. This contained B5 salts and vitamins, 3 mg/l benzyl adenine, 1 mg/l zeatin, and 1% sucrose. It was supplemented with 500 mg/l carbenicillin and 50 mg/l kanamycin sulfate. This medium was solidified using 0.7% Phytagar. Under lighted conditions (16-8 light-dark cycles at 24°C, $100\ \mu Em^{-2}s^{-1}$) the tissue began to develop green callus. Under non-selective conditions without kanamycin sulfate) numerous shoots form on this medium which can be propagated and rooted. Under selective conditions green callus and shoot formation is evident, but greatly reduced.

Under these selective conditions successful transformation events leading to kanamycin-resistant material will grow and may be scored for frequency. To assure that the primary selection pressure is for kanamycin resistance and not nutrient scavenging or insensitivity to inhibitors released from dying tissue, the explants were re-plated on the identical medium every 7-10 days.

Shoots and callus growing on the kanamycin-containing medium may be tested for the expression of the neomycin phosphotransferase gene using an assay described by Reiss et al., Gene (1984) 30:211-218. This employs polyacrylamide gel electrophoresis to separate the enzyme from background proteins. Enzyme

activity is detected *in situ* by ATP mediated phosphorylation of kanamycin using γ-^{32}P-labelled ATP. The product of the reaction is blotted onto P81 ion exchange paper which is then treated (45 min at 65°C) with Proteinase K (1 mg/l, Sigma Chemicals) in 1% sodium dodecyl sulfate. This treatment removes much of the background radioactivity on the paper associated with ^{32}P-labelled proteins. The phosphorylated kanamycin remains intact during the treatment. This product is then detected by autoradiography and may be quantified by scintillation counting. An example of such an assay performed on transformed, kanamycin resistant *Brassica napus* tissue is shown in Fig. 1. The phenotype of the transformant is kanamycin resistance. The level of resistance in a *Brassica* A281x200 transformant is shown in Fig. 2.

Example III

Transformation may be conducted using *Agrobacterium tumefaciens* in the presence of a feeder cell layer. This may be advantageous both to help the *Agrobacterium*-treated tissue recover and for stimulation of transformation activity. In this embodiment the tissues are prepared as described in Example II, but then are transferred, after dipping into *Agrobacterium* and blotting, onto Petri plates containing feeder cells of *Nicotiana tabacum* (tobacco) suspension cells. The feeder plates are prepared by pipetting 1.0 ml of a stationary phase tobacco suspension culture onto B5 medium containing 1 mg/l of both 2,4-D and kinetin with vitamins as described above. The medium is solidified using 0.6% agar. The feeder plates are produced 24-48 hours prior to use, and the excised *Brassica* tissue may be pre-incubated on the feeder plate by placing a sterile Whatman 3 mm filter paper on top of the feeder layer and arranging the excised *Brassica* tissue on this 24 hours prior to *Agrobacterium* treatment.

After dipping in Agrobacterium tumefaciens (A281x200 or similar strain) the Brassica explants are returned to the feeder plates for a further 24-48 hours. After this time they are transferred to B5 medium containing 1 mg/l kinetin, 1 mg/l, 2,4-D and 500 mg/l carbenicillin in agarized medium (0.6%). All other steps are identical to those described in Example II.

Example IV

This transformation method can also be applied effectively to hypocotyl explants rather than leaf explants. All procedures for transformation of the hypocotyl explants are identical to those described above for leaf discs; however, the preparation of hypocotyl material differs.

Seeds of Brassica napus cv Westar were surface sterilized in a 1% sodium hypochlorite solution containing 200 µl of "Tween 20" surfactant per 500 ml of sterilant solution. After 20 minutes soaking in the sterilant the seeds were washed (4 times) with sterile distilled water and planted in sterile plastic boxes 7 cm wide, 7 cm long, and 10 cm high (Magenta) containing 50 ml of 1/10 concentrated B5 medium (Gamborg, Miller and Ojima, Experimental Cell. Res. (1968) 50:151-158) containing no growth substances and solidified with 0.6% agar. The seeds germinated and were grown at 23-25°C in a 16-8 hour light-dark cycle with light intensity approximately 100$\mu Em^{-2}s^{-1}$. After 5 days the seedlings were taken under sterile conditions and the hypocotyls excised and cut into pieces of about 4 mm in length. These hypocotyl segments were then treated with all the same procedures applied to leaf disc explants in Example II.

Example V

Seeds of Brassica napus cv. Westar were soaked in 95% ethanol for 4 minutes. They were sterilized in 1% solution of sodium hypochlorite with 50 µl of "Tween 20" surfactant per 100 ml sterilant solution. After soaking for 45 minutes, seeds were rinsed 4 times with sterile distilled water. They were planted in sterile plastic boxes 7 cm wide, 7 cm long, and 10 cm high (Magenta) containing 50 ml of 1/10th concentration of MS (Murashige minimal organics medium, Gibco) with added pyridoxine (50 µg/l), nicotinic acid (50 ug/l), glycine (200 ug/l) and solidifed with 0.6% agar. The seeds germinated and were grown at 22°C in a 16-8 hour light-dark cycle with light intensity approximately 65 $\mu Em^{-2}s^{-1}$. After 5 days the seedlings were taken under sterile conditions and the hypocotyls excised and cut into pieces of about 4 mm in length. The hypocotyl segments were placed on a feeder plate described in Example III or without the feeder layer on top of a filter paper on the solidified B5 0/1/1 medium. This was done 24 hours prior to Agrobacterium treatment.

Agrobacterium tumefaciens (strains A281 x 767 and EHA101 x 767) were prepared by incubating a single colony of Agrobacterium in MG/L broth at 30°C. Bacteria were harvested 16 hours later and dilutions of 10^8 bacteria per ml were prepared in MG/L broth. Hypocotyl segments were inoculated with bacteria by placing in Agrobacterium suspension and allowed to sit for 30-60 minutes, then removed and transferred to Petri plates containing B5 0/1/1 medium described above. The plates were incubated in low light at 22°C. The co-incubation of bacteria with the hypocotyl segments took place for 24-48 hours. The hypocotyl segments were removed and placed on B5 0/1/1 containing 500 mg/l carbenicillin (kanamycin sulfate at 10, 25, or 50 mg/l was sometimes added at this time) for 7 days in continuous light (approximately 65 $\mu Em^{-2}s^{-1}$) at 22°C.

They were transferred to B5 medium with 3 mg/l BAP and 1 mg/l zeatin as described in Example III. This was supplemented with 500 mg/l carbenicillin, 10, 25, or 50 mg/l kanamycin sulfate, and solidified with 0.6% Phytagar (Gibco). Thereafter explants were transferred to fresh medium every 2 weeks.

After 1 month green shoots developed from green calli which were selected on media containing kanamycin. Shoots continued to develop for 3 months. The shoots were cut from the calli when they were at least 1 cm high and placed on B5 medium with 1% sucrose, no added growth substances, 300 mg/l carbeniclin, and solidified with 0.6% phytagar. The shoots continued to growth and several leaves were removed to test for neomycin phosphotransfersase II (NPTII) activity. Shoots which were postiive for NPTII activity were placed in Magenta boxes containing B5 medium with 1% sucrose, 2 mg/l indolebutyric acid, 200 mg/l carbenicillin, and solidified with 0.6% Phytagar. After a few weeks the shoots developed roots and were transferred to soil. The plants were grown in a growth chamber at 22°C in a 16-8 hours light-dark cycle with light intensity 220 $\mu Em^{-2}s^{-1}$ and after several weeks were transferred to the greenhouse.

Leaves were harvested, frozen in liquid nitrogen and DNA extracted (Dellaporta et al., Pl. Molec. Biol. Reporter (1983) 1:19-21). Southern analysis (Maniatis et al., "Molecular Cloning", Cold Spring Harbor Press) confirmed proper integration of the T-DNA.

Using the method described above 2% of the hypocotyl segments produced shoots which were positive for NPTII activity.

Frequency of hypocotyl explants producing NPTII positive shoots

Date	Agrobacterium Strain	
	EHA101pCGN767	A281 pCGN767
1	1/59 2%	1/59 2%
2	1/57 2%	
3	1/59 2%	1/54 2%
	1/62 2%	1/60 2%
	1/64 2%	1/59 2%
	2/58 3%	1/60 2%
	1/58 2%	1/59 2%
	2/61 3%	1/59 2%

Transgenic plants have been also obtained from hypocotyl segments of *B. napus* cultivars Westar, Viking and Bridger cocultivated with *Agrobacterium* strains EHA101 and K61 containing other constructs with other plant genes. The system is repeatable with different *Agrobacterium* strains, constructs, and *Brassica* genotypes.

Example VI

The shoot regeneration frequency from hypocotyl segments was increased at least two-fold by removing kinetin from the cocultivation and callusing medium (B5 0/1/1 to B5 0/1/0).

Frequency of hypocotyl explants producing at least one shoot

Date	*B. napus* cultivar	Callusing medium B5 0/1/0		B5 0/1/1	
1	Westar	13/22	59%	3/24	12%
2	Westar	12/20	60%	2/28	7%
3	Westar	17/23	74%	3/24	12%
4	Westar	26/29	90%	12/28	43%
5	Viking	95/100	95%	53/102	52%
6	Bridger	58/101	57%	13/77	17%

This change provides an increased number of transformed shoots recovered from hypocotyl explants which were inoculated with Agrobacterium.

The above results demonstrate that Brassica species can be transformed efficiently, whereby foreign genes may be integrated into the plant genome and expressed, providing novel phenotypic properties. Thus, Brassica species can be transformed and are shown to be capable of utilizing genes where the transformed cells may be regenerated into plants which provide for expression of the novel phenotype. By virtue of the high transformation efficiency, successful transformations can be achieved within reasonable time periods and without unduly repetitive procedures.

All patents, other publications, and patent applications mentioned above are illustrative of the skill of those skilled in the art to which the invention pertains. Each patent, other publication and patent application is herein individually incorporated by reference in the same location and to the same extent as if each patent, other publication, or patent application had been individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

ANNEX M3

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 5, line 16-17 of the description[1]

A. IDENTIFICATION OF DEPOSIT[2]

Further deposits are identified on an additional sheet ☐[3]

Name of depositary institution[4]

American Type Culture Collection

Address of depositary institution (including postal code and country)[4]

12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit[5] 20 May 1987	Accession Number[6] 53621

B. ADDITIONAL INDICATIONS[7] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE[8] (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS[8] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later[9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau[10]

was

(Authorized Officer)

Form PCT/RO/134 (January 1981)

(January 1985)

WHAT IS CLAIMED IS:

1. Transformed Brassica species cells having a DNA construct resulting from in vitro joining of at least two fragments, wherein said fragments comprise:

(1) a transcription initiation region functional in said Brassica;

(2) a DNA sequence comprising an open reading frame having an initiation codon at its 5' terminus or a sequence complementary to an endogenous transcription product;

(3) a transcription termination region functional in said Brassica;

(4) a right border of T-DNA;

(5) a structural gene capable of expression in said Brassica providing for selection of transformed Brassica cells;

wherein said fragments provide an expression cassette capable of expression in said Brassica cells.

2. Cells according to Claim 1, wherein said DNA is an open reading frame.

3. Cells according to Claim 2, wherein said open reading frame imparts a phenotypic property to said cells.

4. Cells according to Claim 1, wherein said construct comprises at least one T-DNA border.

5. Cells according to Claim 1, wherein said Brassica is napus or campestris.

6. Cells according to Claim 1, wherein said transcription initiation region is the 35S region of cauliflower mosaic virus.

7. A method for transforming Brassica cells to produce Brassica plants, said method comprising:

co-cultivating Brassica cells with A. tumefaciens comprising a plasmid comprising an insertion sequence resulting from the in vitro joining of a transcription cassette to at least the right T-DNA border and a marker which provides for selection of cells containing said marker, whereby said Brassica cells are transformed with said insertion sequence which becomes integrated into the plant cell genome;

transferring said transformed Brassica cells to callus inducing media containing at least one auxin and selective for cells comprising said marker to produce callus from said transformed cells;

transferring said callus to regeneration media containing less than about 2% sucrose or organic caloric equivalent to produce shoots; and

transferring said shoots to a growing medium to produce plants.

8. A method according to Claim 7, wherein said Brassica cells are hypocotyl cells.

9. A method according to Claim 8, wherein said hypocotyl cells are in the form of stem segments.

10. A method according to Claim 7, wherein said callus inducing media is free of cytokinins.

11. A method according to Claim 7, wherein said regeneration media contains about 1% sucrose.

12. A method according to Claim 7, wherein said A. tumefaciens is a disarmed strain.

13. A method according to Claim 7, wherein said callus inducing medium has about 1 mg/l of an auxin and from about 0 to 1 mg/l of cytokinin and said regeneration media contains about 1% sucrose.

14. A method according to Claim 7, wherein said *Brassica* is of the species *campestris* or *napus*.

15. A *Brassica* plant comprising cells according to Claim 1.

16. A *Brassica* plant according to Claim 15, wherein said *Brassica* is of the species *campestris* or *napus*.

17. A cell culture of cells according to Claim 1.

18. A cell culture of cells according to Claim 17, wherein said culture includes a selective agent.

19. A plant produced according to the method of Claim 7.

20. A plant according to Claim 19, wherein said transcription cassette comprises the cauliflower mosaic virus 35S promoter.

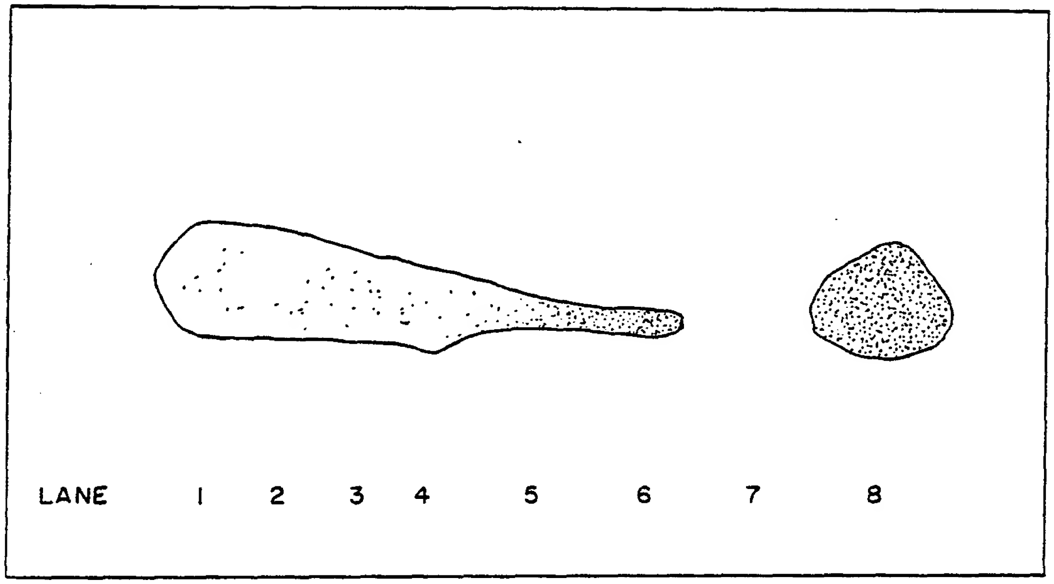


FIG. I

FIG. 2

SENSITIVITY OF TRANSFORMED AND UNTRANSFORMED TISSUE TO KANAMYCIN

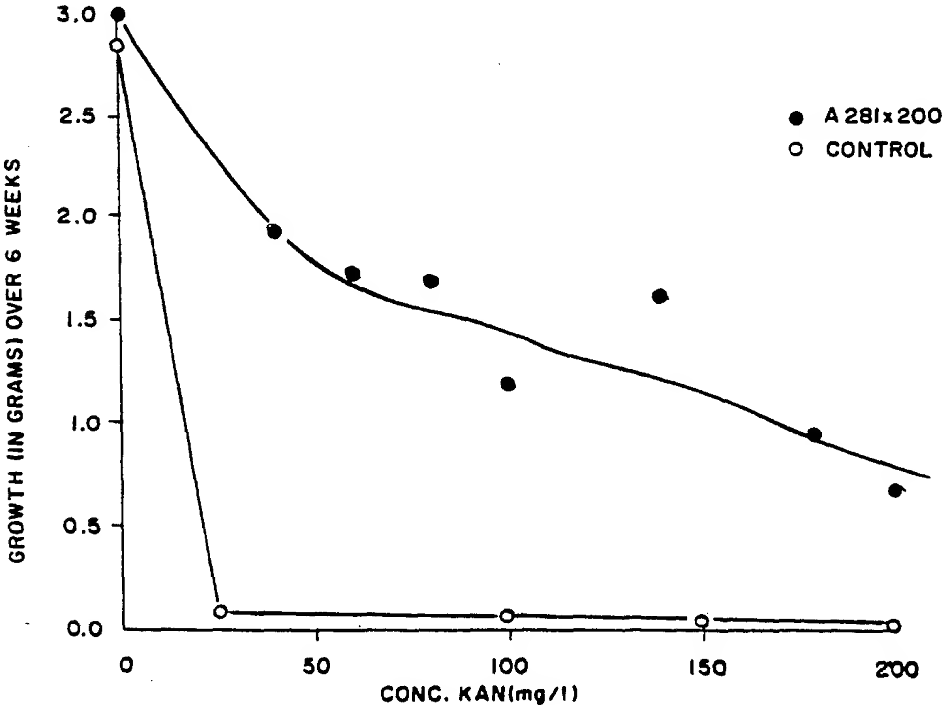


FIG. 3A

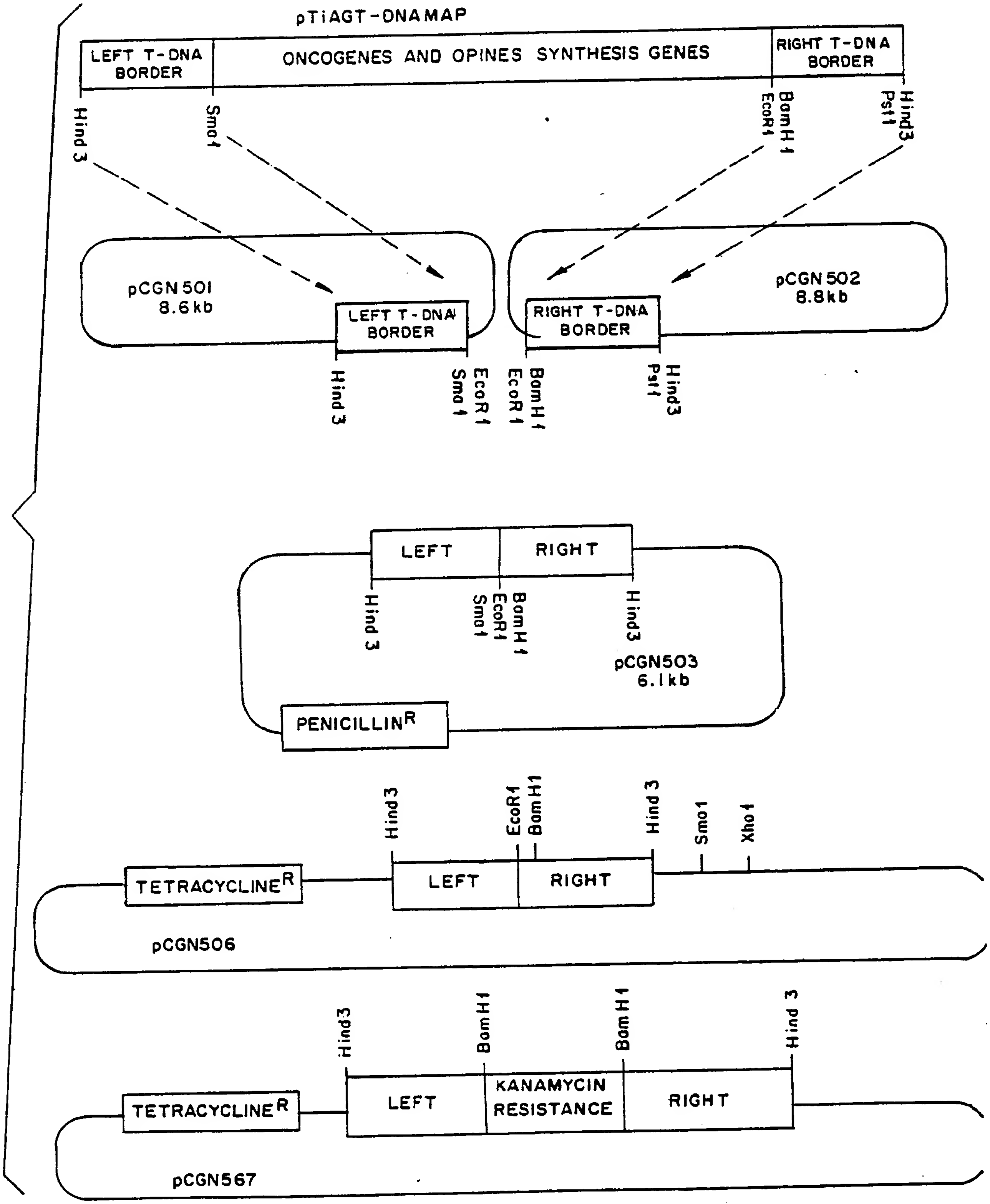
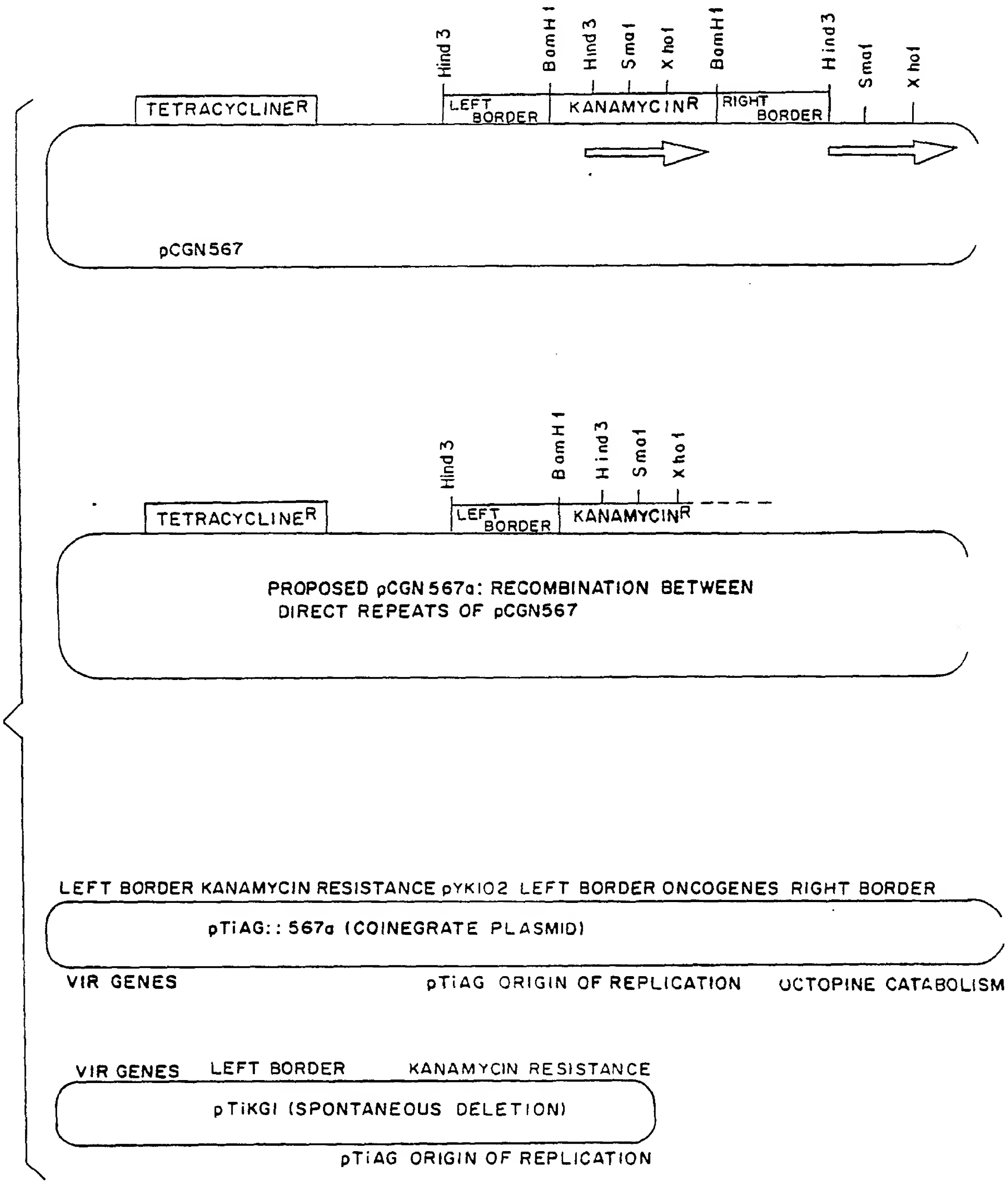

pTiAGT-DNAMAP
LEFT T-DNA BORDER
ONCOGENES AND OPINES SYNTHESIS GENES
RIGHT T-DNA BORDER
Hind 3
Sma1
BamH1
EcoR1
Hind3
Pst1
pCGN501
8.6kb
LEFT T-DNA BORDER
Hind 3
Sma1
EcoR1
RIGHT T-DNA BORDER
EcoR1
BamH1
Pst1
Hind3
pCGN502
8.8kb
LEFT
RIGHT
Hind 3
Sma1
EcoR1
BamH1
Hind3
pCGN503
6.1kb
PENICILLIN^R
Hind3
EcoR1
BamH1
Hind 3
Sma1
Xho1
TETRACYCLINE^R
LEFT
RIGHT
pCGN506
Hind3
BamH1
BamH1
Hind 3
TETRACYCLINE^R
LEFT
KANAMYCIN RESISTANCE
RIGHT
pCGN567

FIG. 3B


Hind 3
BamH1
Hind 3
Sma1
Xho1
BamH1
Hind 3
Sma1
Xho1
TETRACYCLINE^R
LEFT BORDER
KANAMYCIN^R
RIGHT BORDER
pCGN567
Hind 3
BamH1
Hind 3
Sma1
Xho1
TETRACYCLINE^R
LEFT BORDER
KANAMYCIN^R
PROPOSED pCGN567a: RECOMBINATION BETWEEN DIRECT REPEATS OF pCGN567
LEFT BORDER KANAMYCIN RESISTANCE pYK102 LEFT BORDER ONCOGENES RIGHT BORDER
pTiAG::567a (COINEGRATE PLASMID)
VIR GENES
pTiAG ORIGIN OF REPLICATION
OCTOPINE CATABOLISM
VIR GENES
LEFT BORDER
KANAMYCIN RESISTANCE
pTiKGI (SPONTANEOUS DELETION)
pTiAG ORIGIN OF REPLICATION

INTERNATIONAL SEARCH REPORT

International Application No PCT/US87/01205

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): C12N 15/00 C12N 5/00 C12N 5/02 C12N 1/00 A01H 1/04
US CL. 435/172.3 435/240 435/241 435/317 800/1

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
US	435/172.3 240 241 317 800/1

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

Computer Search CAS, Biosis 1969 to present:
Brassica, Agrobacterium

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X Y	Biologia Plantarum, Volume 25, issued November 1983, (Prague, Czechoslavakia), M. Ondrej, "Detection of nopaline dehydrogenase and lysopine dehydrogenase activities in crown gall tumors of different plant species," pages 378-384, see page 379 in particular.	1-4,17 5-16, 18-20
X Y	Journal of Genetics, Volume 65, issued January 1986, (Bangalore, India) Mathews et al., "Transformation of Brassica juncea by Agrobacterium tumefaciens harboring plasmid pTiT37 and its 'rooty' mutant pTiT37.14a/a," pages 37-44, see pages 38-40 in particular.	1-4,17 5-16, 18-20
X Y	Biological Abstracts, Volume, 81, Number 8, issued 15 April 1986, (Philadelphia, Pennsylvania, USA), Ooms et al., "Genetic manipulation in cultivars of oilseed rape Brassica napus using Agrobacterium," see page 851, column 2, the abstract number 76080, Theor. Appl. Genet., 1985, 71(2): 325-329.	1-5,17 6-16, 18-20

* Special categories of cited documents: [15]

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2] 30 JULY 1987	Date of Mailing of this International Search Report [2] 12 AUG 1987
International Searching Authority [1] ISA/US	Signature of Authorized Officer [20] David T. F David T. Fox

Form PCT/ISA/210 (second sheet) (May 1986)

International Application No.
PCT/US87/01205

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
Y	Journal of Molecular and Applied Genetics, Volume 2, issued June 1984, (New York, New York, USA), Koziel et al., "A cauliflower nosaic virus promoter directs expression of kanamycin resistance in morphogenic transformed plant cells," pages 549-562, see page 560 in particular.	6,12,18, 20
Y	Zeitschrift fur Pflazenphysiologie, Volume 92, issued January 1980, (Stuttgart, West Germany), G. Stringham, "Regeneration in leaf-callus cultures of haploid rapeseed," pages 459-462, see page 460 in particular.	10,13-16
Y	Biological Abstracts, Volume 75, Number 7, issued April 1, 1983, (Philadelphia, Pennsylvania, USA), Dietert et al., "Effects of genotype on in vitro culture in the genus Brassica," see page 5385, column 1, the abstract number 52464, Plant Sci. Lett., 1982, 26 (2/3): 233-240.	8,9, 14-16
Y	Flick et al., "Organogenesis," Volume published 1983, by Evans et al., (New York, New York, USA), pages 25-26, see pages 25 in particular.	10, 13-16
Y	Annals of Botany, Volume 54, issued June 1984, (London, England), Lazzeri et al., "In vitro shoot regeneration from seedling root segments of Brassica oleracea and Brassica napus cultivars," pages 341-350, see page 344 in particular.	11, 13-16
A	Physiologica Plantarum, Volume 61, issued March 1984 (London, England), K. Glimelius, "High growth rate and regeneration capacity of hypocotyl protoplasts in some Brassicaceae," pages 38-44, see pages 39 and 42 in particular.	11, 13-16

International Application PCT/US87/ 01205

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X Y	Chemical Abstracts, Volume 103, Number 21, issued November 25, 1985, (Columbus, Ohio, USA), Jiang et al., "T-DNA transfer and teratome induction on Brassica oleracea L," see page 427, column 2, the abstract number 175561f, Kexue Tongbao, 1985, 30 (6): 826-828.	1-4,17 5-16, 18-20

V. ☐ **OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE** [10]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers , because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers .... , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

VI. ☐ **OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING** [11]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, 16 with indication, where appropriate, of the relevant passages 17	Relevant to Claim No 18
X Y	Chemical Abstracts, Volume 103, Number 11, issued September 16, 1985, (Columbus, Ohio, USA), Mathews et al., "Regeneration of shoots from Brassica juncea (Linn) Czern and Coss cells transformed by Agrobacterium tumefaciens and expresion of nopaline dehydrogenase genes," see page 378, column 1, the abstract number 85227x, Plant Sci., 1985, 39(1): 49-54.	1-4,17 5-16, 18-20
X Y	Plant Physiology, Volume 75, Supplement 1, issued December 1984, (Rockville, Maryland, USA) Moloney et al., "Incorporation of foreign genes into Brassica using co-cultivation with Agrobacterium spp.," see page 86.	1-5,17 6-16, 18-20
X Y	Physiologica Plantarum, Volume 64, issued March 1985, (London, England), Laser et al., "Transformation of protoplasts from Brassica campestris with Agrobacterium tumefaciens," see page 13A.	1-5 6-20
X Y	Plant Physiology, Volume 67, issued June 1981, (Rockville, Maryland, USA), Galsky et al., "Crown-gall tumors possess tumor-specific antigenic sites on their cell walls," pages 1195-1197, see page 1195 in particular.	1-4 5-20
X Y	Plant Cell Reports, Volume 4, issued December 1985, (New York, New York, USA), Holbrook et al., "Brassica crown gall tumorigenesis and in vitro of transformed tissue", pages 329-332, see page 330 in particular.	1-5,17 6-16, 18-20
Y	The EMBO Journal, Volume 4, issued August 1985, (Oxford, England), Bevan et al., "Expression of tobacco mosaic virus coat protein by a cauliflower mosaic virus promoter in plants transformed by Agrobacterium", pages 1921-1926, see page 1925 in particular.	6,12,18, 20

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(51) International Patent Classification ^{6} :
A01H 5/00, 5/08, C12N 5/14, 15/64, 15/82

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(54) Title: *AGROBACTERIUM TUMEFACIENS* TRANSFORMATION OF *MUSA* SPECIES

(57) Abstract

Methods are provided for transforming *Musa* plants. In particular, methods for wounding meristematic *Musa* plant tissue to facilitate access of *Agrobacterium tumefaciens* comprising genetically-engineered T-DNA is provided. The methods may be used to transform the plant to produce pharmaceutical products or to alter the phenotypic trait of the fruit of the plant.

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AGROBACTERIUM TUMEFACIENS TRANSFORMATION OF *MUSA* SPECIES

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to methods for transforming *Musa* species such as bananas or plantains using *Agrobacterium tumefaciens*.

2. Description of the Prior Art

Bananas and plantains are perennial giant herbs belonging to the Genus Musa; their fruits are the fourth most important food in the developing world. Approximately 10% of the world's production of bananas (over 9 million tons in 1990, at a value of over US $4 billion) enters the export market to generate an important source of income for tropical and subtropical regions. In light of the fact that *Musa* spp. make such an enormous contribution to food security and also provide export revenue in developing countries, it is a remarkable paradox that these crops have never benefited from traditional crop breeding. Production around the world is entirely dependent on unimproved clones that were often collected from nature, domesticated and maintained by clonal propagation. In terms of important crops for which biotechnology offers the possibility for dramatic genetic improvement, there are few opportunities as open for novel approaches as *Musa* spp.

There is general agreement that edible bananas originated from the two species, *M. accuminata* and *M. balbisiana*. Edible bananas have three different ploidy levels: $2N=22$, $3N=33$ and $4N=44$. They have been propagated vegetatively for hundreds of years, with somatic mutations providing variability. Triploids are the most numerous and widely utilized cultivars (including dessert

bananas used in world export trade). Efforts to breed *Musa* using conventional methods are fraught with obstacles, including low fertility, levels of ploidy, and lack of genetic variability. Since almost all accepted cultivars are seedless, sterile, clonally-propagated plants, conventional breeding efforts must begin with unimproved material that has been poorly characterized for genetic traits. It would be a major advantage if it were to be possible to make genetic improvements in the currently accepted seedless cultivars that are in production as food crops; this manuscript describes a system for reproducible and rapid genetic transformation of *Musa* that will make this possible.

Although traditional breeding has been slow for *Musa* spp., application of the tools of plant tissue culture have been significant value to crop improvement. Embryo rescue, cell suspension cultures, and related cell culture techniques have been used in research activities to overcome limitations in crop breeding, with resultant selection of genetic variants with new phenotypes. Plant cells grown in unorganized (callus, cells and protoplasts) cultures undergo ubiquitous genetic change or somaclonal variation. While this genetic variability is useful in creating new germplasm, it is a distinct negative feature in the clonal propagation of desired cultivars for which genetic uniformity is necessary. This has led to development of shoot-tip culture protocols which are now widely in use in developed and developing countries for *Musa* multiplication. Characteristically, these micropropagation procedures require only short periods of exposure of the plant tissues to growth in an undifferentiated state, and have a very low level of somaclonal variation among progeny.

In the development of the transformation system described below, we have attempted to devise a system that would mimic the widely used shoot micropropagation procedures now in use commercially. Our goal was to make available a system that would be useful for targeted genetic modification of existing, valuable cultivars with the least possible probability of introducing unanticipated somaclonal variability.

Virulent strains of the gram negative soil bacterium *Agrobacterium tumefaciens* are known to infect dicotyledonous plants and certain monocotyledonous plants. The tumor-inducing agent in the *A. tumefaciens* is a plasmid that functions by transferring some of its DNA into its host plant's cells. This plasmid (the Ti plasmid), and the virulence of the various strains of *A. tumefaciens* is determined in part by the vir region of the Ti plasmid, which is responsible for mobilization and transfer of T-DNA. Foreign genes may be mobilized and delivered to a susceptible host via the Ti plasmid.

The Ti plasmid can be used as a vector for the genetic engineering of host plants. In most cases the native *A. tumefaciens* Ti plasmid is modified to create a disarmed plasmid, that is, one that does not cause tumor formation or disease.

It has generally been assumed that the host range of *A. tumefaciens* was limited to dicotyledous species. There has been limited success in transformation of some monocotyledous plants such as *Gramineae* (*see* U.S. Pat. Nos. 5,187,073 and 5,177,010). Hooykaas-Van Slogteren et al., *Nature*, 311, 763 (1984), reported the production of small swellings at wound sites infected with *A. tumefaciens* in monocotyledous species of the Liliaceae and Amaryllidaceae families. Hernalsteens, et al. reported [*EMBO Journal*, 3, 3039 (1984)] that cultured stem fragments of the monocotyledon *Asparagus officinalis*, a member of the family Liliaceae, infected with *A. tumefaciens* strain C58 developed tumorous proliferations. DeCleene and DeLey in *The Botanical Review*, 42, 389 (1976) teach that monocots of the orders Liliales and Arales are susceptible to infection with *A. tumefaciens*, but that monocotyledons in general are unsusceptible to *A. tumefaciens* infection. It has been noted [Potrykus, *Bio/Technology* 8:515 (1990)] that monocot transformation is difficult with *Agrobacterium* because these species do not show the wound response characteristic of dicots (which are competent for activating *vir* gene expression in the bacterium, transformation, and regeneration of tissues in which the introduced genes are integrated into the plant cell chromosome). There is insufficient data available to predict the extent to which

other monocots can be made susceptible to *Agrobacterium*. Chilton [*Proc. Natl. Acad. Sci.* 90:3119 (1993)] noted that host plant chemistry is important for the delicate chemical signalling between bacteria and plant cells. This situation has further complexity because of differences in agroinfection efficiency between different Ti plasmid vectors. Chilton teaches that there is a great deal of uncertainty in the transformation of monocots which makes it impossible to predict in advance that *Musa* can be transformed. Because of this uncertainty, it cannot be predicted in advance if the bacterial gene transfer will occur in other untested monocots, such as *Musa*.

No one has reported the transformation of any member of the monocotyledonous *Musa* family (such as bananas and plantains) by infection with *A. tumefaciens*. It has heretofore been generally thought that *Musa* species are not susceptible to transformation by *A. tumefaciens*. In fact, the prior art teaches that *A. tumefaciens* cannot be used to transform *Musa*. [*Biotech. and Devel. Monitr.*, **14**:14-16 (1993)].

There is a need to develop a method for effecting genetic transformation of *Musa*. The ability to insert foreign genes into these popular tropical plants would be of great importance since it would allow attempts to create disease and pest resistance and altered fruit phenotypes *via* genetic engineering.

These and other advantages of the present invention will become apparent from the following detailed description.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide methods for transforming *Musa* plants. It is also an additional object of the present invention to provide transformed *Musa* plants.

Accordingly, the present invention provides a method for transforming a *Musa* plant, said method comprising: wounding meristematic tissue from a *Musa* plant to generate a wounded *Musa* plant tissue and to facilitate access of *Agrobacterium tumefaciens* to *Musa* plant cells competent for transformation and regeneration; and applying to said wounded *Musa* plant tissue at least one transformation competent *Agrobacterium tumefaciens* to transform said *Musa* plant, wherein said at least one transformation competent *Agrobacterium tumefaciens* harbors at least one Ti plasmid, wherein said at least one Ti plasmid comprises at least one genetically engineered T-DNA to effect transformation of said *Musa* plant.

The present invention also provides a method for transforming *Musa* plants wherein the at least one genetically engineered T-DNA further comprises at least one second gene selected from the group consisting of genes which code for selection agent resistance, genes which code for at least one screenable marker, and combinations thereof.

The present invention also provides a method for transforming *Musa* plants, wherein the at least one genetically engineered T-DNA further comprises at least one gene which codes for selection agent resistance, and wherein the method further comprises regenerating the transformed *Musa* plant tissue in the presence of at least one selection agent responsive to the at least one gene which codes for selection agent resistance so as to select for resistant tissue transformed with the at least one genetically engineered T-DNA.

The present invention also provides a *Musa* plant (and progeny thereof) which includes cells which comprise in its genome at least one gene selected from the group consisting of at least one gene which codes for at least one polypeptide non-native to the *Musa* plant, at least one gene which codes for

at least one polypeptide native to the *Musa* plant, at least one gene which codes for altered gene expression of at least one native *Musa* gene, and combinations thereof.

Also provided is a method for transforming *Musa* plants with at least one pharmaceutical selected from the group consisting of hepatitis B surface antigen, Norwalk virus capsid protein, insulin, interleukins, growth hormone, erythropoietin, G-CSF, GM-CSF, hPG-CSF, M-CSF, Factor VIII, Factor IX, tPA, insulin, and combinations thereof. Also provided are plants transformed with at least one pharmaceutical.

Also provided is a method for transforming *Musa* plants with at least one gene that changes the phenotypic trait of the fruit of the plant. Also provided are transformed plants that have fruit having an altered phenotypic trait.

Also provided is a method for transforming *Musa* plants so as to confer herbicide and/or disease resistance to the plants.

Also provided is a method for transforming a *Musa* plant wherein the transformed *Musa* plant is grown for a sufficient time to identify the presence of chimeric features, producing nonchimeric tissue by dividing the transformed *Musa* plant into segments which have at least one meristem which can regenerate into an intact plant and which have cells that are uniformly transformed to produce nonchimeric tissue, and growing the nonchimeric tissue into a nonchimeric plant.

These and other objects and advantages are described in the following Detailed Description.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts NPT-II dot-blot assays of individual regenerant plants following *Agrobacterium*-mediated genetic transformation. Each well (spot) contains an extract of leaf tissue from an individual plant. Rows A, B, and C all contain extracts from putative banana transformants recovered after selection on 100 mg/l kanamycin sulfate. Row D contains extracts from non-transformed control banana plants. Samples E-1 and E-2 are non-transformed and transformed tobacco plants, shown for comparison. Variability in intensity of the reaction among different putative regenerants is attributed to the random chromosomal insertion of the T-DNA into the banana chromosomes, with concomitant variability in levels of NPT-II expression.

Figure 2 depicts β-glucuronidase (GUS) activity in extracts of leaf tissue from individual plants derived from *Agrobacterium*-mediated transformation of banana meristematic tissues. Individual plants are identified by number. GUS activity is measured fluorimetrically as the rate of molar conversion of the substrate [4-methyl-umbelliferyl-β-D-glucuronide (MUG)] to the product [4-methyl umbelliferone (4-MU)] by the enzyme β-glucuronidase. This activity is expressed as moles of product produced per minute per mg of total protein contained in the assay mixture.

Figure 3 depicts an autoradiograph of a Southern blot of genomic DNA from negative control (non-transformed), positive control (transgenic tobacco plants harboring *NTP-II*), and banana plants transformed by *Agrobacterium* which were regenerated on 100 mg/l kanamycin. Ten micrograms of each genomic DNA were *EcoRI* digested and were loaded onto separate lanes. These DNAs were hybridized against a 1.0 kb *PstI* fragment of *NPT-II*. DNA from plants (transgenic banana and transgenic tobacco) which exhibit NPT-II activity (lanes 1 thru 7 and lane 9), demonstrated hybridization to the *NPT-II* fragment. The non-transformed (negative control) banana plant (lane 8) demonstrated no detectable hybridization.

Figure 4 depicts an autoradiograph of a Southern blot of genomic DNA from positive control (*Agrobacterium* genomic DNA), negative control (non-transformed banana plants), and banana plants transformed by *Agrobacterium* which were regenerated on 100 mg/l kanamycin. One microgram of the *Agrobacterium* DNA and 10 ug of the plant DNAs were *EcoRI* digested and were loaded onto separate lanes. These DNAs were hybridized against a 1.0 kb *Bam HI/BaglII* fragment containing *vir B*. DNA from *Agrobacterium* demonstrated hybridization to the *vir B* fragment (lane 1). Neither the non-transformed (negative control) banana plant (lane 2), nor the transgenic banana plants (lanes 3-5) demonstrated detectable hybridization.

DETAILED DESCRIPTION OF THE INVENTION

The method of the present invention is for the generation of stable, transformed *Musa* species. The term *Musa* includes bananas and plantains. Suitable *Musa* tissues for transformation include meristematic tissue such as, but not limited to, apical meristem, adventitious meristem, and corm tissues of growing banana or plantain shoot cultures. Suitable tissue must also be capable of regeneration into at least one intact plant. Lateral or axial meristematic tissue may also be suitable.

The method involves wounding meristematic tissue (to facilitate infection by *Agrobacterium tumefaciens*) by techniques such as, but not limited to, disection (*e.g.*, longitudinal bisection), cutting, puncturing, and/or micro-particle bombardment. Wounding may be enhanced in effectiveness by micro-particle bombardment of dissected tissue. Micro-particle bombardment may be conducted by any technique known to those skilled in the art. Such techniques include, but are not limited to, tungsten or gold micro-particle bombardment. It is anticipated that wounding not only provides exposed tissue which is competent for transformation by *Agrobacterium tumefaciens*, but also stimulates the production of compounds which induce the virulence of the bacterium.

After wounding, meristematic tissues may be incubated for a suitable incubation time period such as about one to about eight, preferably about two to about six and most preferably about four days at a suitable incubation temperature and suitable illumination regime. Suitable incubation temperatures are about 25 °C to about 29 °C, preferably about 26 °C to about 28 °C, and most preferably at about 27 °C. Suitable illumination regimes are about 14 hours to about 18 hours, and preferably about 15 hours to about 17 hours, and most preferably about 16 hours. In an alternative embodiment the wounded tissue is subjected to *A. tumefaciens* treatment without incubation beforehand.

The method involves applying to the wounded tissue at least one transformation competent *A. tumefaciens* to transform the plant. The selection of the appropriate *A. tumefaciens* strain can be made by those having skill in the

art. An appropriate strain is one which can effectively deliver the gene of interest. Suitable *A. tumefaciens* strains include, but are not limited to, strain LBA4404, C58, and A281, harboring a plasmid which causes gene expression in *Musa*. The *A. tumefaciens* comprises at least one Ti plasmid comprising at least one genetically engineered T-DNA. *A. tumefaciens* may be applied by any technique known to those skilled in the art, such as, but not limited, manual application by fingertip, swabbing, injection and/or by co-cultivation.

Application of *A. tumefaciens* will be for a suitable time period. A suitable incubation time is about 15 to about 60 minutes, preferably about 20 to about 45 minutes, and most preferably about 30 minutes. The genetically engineered T-DNA may carry one or more genes to transform the plant. These may be, for example, at least one first gene which codes for at least one protein non-native to the *Musa* plant, at least one protein native to the *Musa* plant, at least one gene which alters gene expression in the *Musa* plant, and combinations thereof. The genetically engineered T-DNA may also comprise at least one second gene which codes for selection agent resistance, at least one gene which codes for at least one screenable marker, and combinations thereof.

In one preferred embodiment of the present invention, meristematic tissues may be treated with at least one compound which induces the virulence of *A. tumefaciens*. Suitable compounds include, but are not limited to acetosyringone or other plant extracts for inducing *A. tumefaciens* virulence. Other additives may be applied to enhance successful infection, including, but not limited to opines such as octapine, nopaline, and leucinopine. Treatment with such virulence inducing compounds may be conducted by co-cultivating the tissue in media comprising such compounds. The tissues are incubated at a suitable incubation temperature as outlined above for a suitable time period as outlined above. In an alternative embodiment, the co-cultivation is not conducted in the presence of a virulence-inducing compound. In one embodiment, the transformed tissue is planted and allowed to grow. In a preferred alternative embodiment, the transformed tissue is incubated in conditions which allow for selection using

techniques well known to those skilled in the art. For example, the tissue may be incubated in a selective regeneration medium. Selection using at least one antibiotic resistance gene may be used. Insertion of at least one antibiotic resistance gene sequence such as, but not limited to kanamycin, chloramphenicol, neomycin, carbenicillin, hygromycin and combinations thereof may be employed. Also, at least one gene coding for herbicide resistance may be inserted. These include herbicides such as, but not limited to, phosphinothricin, glyphosate, and at least one of the sulfonylureas and combinations thereof. The selective regeneration medium will be comprised of at least one suitable selective ingredient such as appropriate antibiotics for selection of antibiotic-resistant transformants, or at least one herbicide, for herbicide-resistant transformants. The choice of selective agent(s) will depend on the resistance gene transferred into the plant.

Emerging *Musa* regenerates may be transferred to regeneration medium comprising antibiotics and/or herbicides to allow selection of transgenic plantlets. Regeneration media are those media known to those skilled in the art which provide conditions favorable for regenerating plantlets. Suitable regeneration media include, but are not limited to, MS media, as described in Novak, F.J., *et al.*, *Bio/Technology* 7, 154 (February 1989), incorporated herein by reference. Emerging regenerates are allowed to incubate for a sufficient time in the regeneration medium. A sufficient time is that which allows the formation of plantlets.

Growing *Musa* regenerates may then be transferred to a rooting medium. Rooting media are those media known to those skilled in the art which provide conditions favorable for inducing the formation of roots. Suitable rooting media include, but are not limited to, SH medium as described by Novak, F.J., *et al.*, *supra*, incorporated herein by reference. Growing regenerates are allowed to incubate for a sufficient time in the rooting medium. A sufficient time is that which allows the formation of roots.

In a preferred embodiment, *Musa* regenerates are assayed for the presence of the added gene(s), or its product(s), by standard biochemical methods.

These are at least three principal advantages of the method developed for transformation of *Musa* spp. First is its applicability to all commercial cultivars of banana and plantain. In addition, the method allows the introduction of genes into banana or plantain without going through a cell stage that can induce somaclonal variation. In addition, the total process of gene introduction to regeneration of plants is short (less than about two months).

The method of the present invention may be used to transform the *Musa* plant with one or more genes. As used herein "Gene(s)" include, but are not limited to naturally occurring nucleotide sequences or synthetic nucleotide sequences. "Nucleotide sequence" as used herein refers to a chain of natural or modified nucleic acids as commonly recognized by those having skill in the art.

In order to improve the ability to identify transformants, one may desire to employ selectable or screenable marker gene as, or in addition to, the expressible gene of interest. Such genes may encode either a selectable or screenable marker, depending on whether the marker confers a trait which one can select for by chemical means, i.e., through the use of a selective agent such as an herbicide, antibiotic or the like, or whether it is simply a trait that one can identify through observation or testing (e.g., the R-locus trait). Many examples of suitable marker genes are known to the art and can be employed in the practice of the present invention.

Possible selectable markers for use in connection with the present invention include, but are not limited to, a NPT-II gene which codes for kanamycin resistance and can be selected for using kanamycin, G418, etc.; a bar gene which codes for bialaphos resistance, a mutant EPSP synthase gene which encodes glyphosate resistance; etc. Exemplary screenable markers include beta-glucuronidase (GUS), chloramphenicol acetyl transferase (CAT) or an R-locus gene, which encodes a product that regulates the production of anthocyanin

pigments (red color) in host cells. Included within the terms "selectable" or "screenable marker" genes are also genes which encode a secretable marker whose secretion can be detected as a means of identifying or selecting for transformed host cells. Examples include markers which are able to secrete antigen(s) that can be identified by antibody interaction, or an enzyme(s) which can be detected catalytically.

The choice of the particular gene(s) to be delivered to the plant will often depend on the purpose of the transformation. Gene(s) coding for polypeptide(s) non-native to *Musa*, gene(s) coding for polypeptide(s) native to *Musa*, gene(s) which alter gene expression and combinations thereof may be applied to *Musa*. Gene(s) which alter gene expression include, but are not limited, gene(s) which code for at least one ribozyme, gene(s) which code for antisense nucleotides, and gene(s) which operate as a transwitch, such as described in U.S. Patent No. 5,231,020.

The method may be used to transform plants with genes which code for polypeptide(s) non-native to the *Musa* plant. As used herein, "polypeptide" refers to polypeptide or protein. This includes the production of important proteins or other products for commercial use, such as lipase, melanin, pigments, antibodies, hormones, pharmaceuticals such as, but not limited to, interleukins, EPO, G-CSF, GM-CSF, hPG-CSF, M-CSF, Factor VIII, Factor IX, tPA, hGH, receptors, insulin, vaccines, antibiotics and the like. Useful vaccines include, but are not limited to hepatitis B surface antigen and Norwalk virus capsid protein. The genes may also code for fusion proteins. The coding sequences for proteins that can be used are known in the art or can be obtained by standard sequencing techniques. Alternatively, the method may be used to produce an enzyme that is able to convert a natural product to a unique product. This includes, for example, the production of secondary metabolites useful as pharmaceuticals. Alternatively, the method may be used to alter cellular metabolism leading to altered flavor of fruit(s) or altered plant pigmentation or other phenotypic trait(s) of the plant. Such traits include, but are not limited to, visible traits,

environmental or stress related traits, disease related traits, and ripening traits. These include, for example, genes responsible for the synthesis or metabolism of peptides, proteins, fatty acids, lipids, waxes, oils, starches, sugars, carbohydrates, flavors, odors, fragrances, toxins, carotenoid pigments, hormones, cell wall polymers, gene regulatory molecules, flavonoids, storage proteins, phenolic acids, coumarins, alkaloids, quinones, lignins, glucosinolates, tannins, aliphatic amines, celluloses, polysaccharides, glycoproteins and glycolipids.

For instance, an alteration in the production of fatty acids or lipids can be engineered (and fatty acid composition of, e.g., an oil-producing plant thus altered) by blocking synthesis of a specific chain elongation or desaturation enzyme. Also, the synthesis of starch can be altered and sugars altered (and sugar content of, e.g., an edible plant thus altered). Similarly, production of volatile molecules which confer fragrance can be manipulated.

In an alternative embodiment, the method is used for the transformation of plants to add some commercially desirable, agronomically important traits to the plant. Such traits include, but are not limited to, herbicide resistance, increased yields, insect and disease resistance, physical appearance, food content and makeup, etc. For example, one may desire to incorporate one or more genes encoding herbicide resistance. The bar and glyphosate tolerant EPSP synthase genes are good examples. A potential insect resistance gene which can be introduced includes the *Bacillus thuringiensis* crystal toxin gene, which may provide resistance to pests such as lepidopteran or coleopteran.

Genes encoding proteins characterized as having potential insecticidal activity, such as the cowpea trypsin inhibitor (CpTI); may find use as a rootworm deterrent; genes encoding avermectin may prove particularly useful as a corn rootworm deterrent.

In some instances, the transformed *Musa* plant will be chimeric. "Chimeric" refers to a plant having tissue of diverse genetic constitution, or an individual composed of a mixture of genetically different cells. Chimeric plants may, for example, be the result of the insertion of at least one gene which codes

for at least one polypeptide non-native to the *Musa* plant, at least one gene which codes for at least one polypeptide native to the *Musa* plant, at least one gene which codes for altered gene expression of at least one native *Musa* gene, and combinations thereof, into a region of the plant genome which leads to diminished, incomplete, or altered gene expression in only a portion of the cells which are contained in the resultant transgenic plant. In addition, chimeric plants may result from the transformation of a single cell of a meristem in which said meristem (composed of a transformed and nontransformed cells) regenerates into a "chimeric" transformed plant. Chimeric plants can be identified by techniques known to those skilled in the art. These include, but are not limited to enzymatic analyses of the foreign gene product, Southern and Northern hybridization analyses, and histochemical analyses of tissues of different cellular lineages within the same transformed plant. In some cases, chimeric plants can be identified by visual observation of plant features. For example, selected sections of the plant may have stunted or diminished growth.

In some of these cases, known to those of skill in the art, it will be desirable to develop a *Musa* plant that does not have these chimeric features. Generation of a nonchimeric transformed plant from a chimeric transformed plant may be effected by growing a transformed *Musa* plant for a sufficient time to identify the presence of chimeric features, and producing nonchimeric tissue by dividing said transformed chimeric *Musa* plant into segments which have at least one meristem which can regenerate into an intact plant and which have cells that are uniformly transformed to produce nonchimeric tissue, and growing said nonchimeric tissue into a nonchimeric plant. "Sufficient time" as used herein is a time known to those of skill in the art, and includes, but is not limited to about two days to about 45 days, preferably about 10 days to about 30 days, and most preferably about 14 days to about 21 days. "Dividing" as used herein refers to physical cutting, such as by a knife, scalpel or microtome.

The following examples illustrate the teachings of the present invention and are not intended as limiting the scope of the invention.

Example 1

This example describes the transformation of banana using *Agrobacterium tumefaciens*. Neomycin phosphotransferase-II (NPT-II) and Beta-glucuronidase (GUS) were expressed in bananas.

Tissue-culture plantlets of the commercial Cavendish clone 'Grand Nain' (AAA) were micropropagated as shoot-tip cultures. Suspension cultures derived from pro-embryogenic calli were generated and maintained as described by Novak, F.J., et al., *Bio/Technology*, 7, 154-159 (1989), incorporated by reference herein.

Meristematic tips (2-5 mm in size) were excised from in-vitro shoot tip cultures to obtain the meristematic dome with 2-4 leaf primordia and a limited amount of underlying corm tissue; these were then bisected longitudinally. Alternatively, excised corm tissues (which contained numerous adventitious buds) deprived of the apical meristem were cut into slices of 2-3 mm thickness.

The plasmid pBI141 contained the GUS gene under the control of the rice actin 1 (*Act1*) promoter and the NPT-II gene under the control of the NOS promoter. This binary vector was constructed by inserting the Xho1/Xba1 fragment of pAct1 F into Xba1/HindIII digested pBI100. The plasmid pB3G contained both the *bar* and GUS genes under control of the cauliflower mosaic virus 35S promoter.

Longitudinal bisections of apical meristem or adventious bud tissues of growing banana shoot cultures were bombarded with naked micro-particles in a custom designed apparatus at a distance of 10 cm. Bombardment with naked micro-particles prior to incubation of the tissues with *Agrobacterium* further enhanced the percentage of plantlets which grew on selective media. These tissues underwent a three day recovery period. Following recovery, the meristematic or adventitious bud portions were co-cultivated for 30 minutes with a dilute (1:10) overnight culture of the *Agrobacterium* strain LBA4404, harboring pBI141, in the presence of 100μM acetosyringone (AS). After inoculation, the plant tissues were transferred to non-selective S27 regeneration medium as

described by Novak, F.J., *et al.*, *Bio/Technology*, 7, 154-159 (1989), incorporated by reference herein, containing 100 μM AS, and were incubated in the dark at 27°C for four days. Meristematic or advantious bud tissues were then transferred to selective regeneration medium containing 100 mg per liter kanamycin sulfate and 500 mg per liter carbenicillin. Regeneration shoots were transferred to rooting medium as described by Novak, *supra*, containing 100 mg/l kanamycin sulfate and 500 mg/l carbenicillin. Plantlets forming roots on selective medium were assayed for NPT-II activity.

Preliminary experiments were conducted using bisected apical meristems or corm tissues which were incubated with *Agrobacterium* in the presence or absence of acetosyringone (AS). It was found that AS increased the number of regenerating plantlets that were resistant to selective agents, indicating that AS enhanced the *Agrobacterium*-mediated genetic transformation of banana cells.

Putative banana transformants were assayed for the presence of NPT-II activity according to a modified protocol as described by Peng, J. Wen, *et al.*, *Plant Mol. Biol. Rep.*, **11**(1), 38-47, (1993), incorporated by reference herein. In the modification, leaf tissue from plantlets rooting on selective medium were isolated and immediately frozen in liquid N_2. Protein extracts were prepared by homogenizing samples in disposable Kontes tissue grinders in the presence of extraction buffer (50 mM Na phosphate buffer, pH 7.0, 10 mM β-mercaptoethanol, 10 mM EDTA, 0.2% Triton-X100). Samples were centrifuged for four minutes at 4°C, and 50 μl of each extract was added to separate microcentrifuge tubes. Remaining procedures of the assay were performed as stated by Peng, *et al.*

Plants in which NPT-II activities were detected were further analyzed for the presence of GUS enzymatic activity according to standard methods as described by Jefferson, R.A., *Plant Mol. Biol. Rep.*, **5**, 387-405 (1987), incorporated by reference herein.

Genomic DNA was isolated from leaves of plantlets which displayed NPT-II activity according to a CTAB protocol as described by Feinberg, A.P. and Vogelstein, B., *Anal. Biochem.*, 132, 6-13 (1983), incorporated by reference herein. DNA samples (10 μg) were digested with the appropriate restriction enzymes and were electrophoresed through a 0.7% agarose gel, and transferred to a Zeta Probe™ (BioRad, Richmond, CA) nylon membrane according to the suppliers' recommendations. Random primed radiolabled probes were generated from the 1.0 kb EcoRV/SstI GUS fragment. Membranes were hybridized and washed as recommended by the supplier. Results were visualized by autoradiography using standard techniques.

Transformation experiments typically involved 50 micropropagated banana plantlets from which 100 bisected apical meristems and 20 to 40 corm pieces were obtained. Following co-cultivation with *Agrobacterium*, all plantlet regeneration steps included kanamycin sulfate as the selective agent (100mg/l). Seventy percent of the apical meristem pieces formed shoots on micropropagation medium; 50% of these gave vigorous root growth on the selective root regeneration medium. Forty percent of the corm slices formed shoots on the selective micropropagation medium and of these, 40% formed roots on the selective root regeneration medium. Plantlets that formed roots on this selective medium were assayed for enzymatic activity of NPT-II (Figure 1). Approximately 66% of the plants selected demonstrated easily detectable levels of enzymatic activity.

Putative transgenic banana plantlets, selected on the basis of root growth on kanamycin-containing medium and high levels of NPT-II activity were further evaluated for GUS enzymatic activity (Figure 2). Leaf tissue from non-transformed controls demonstrated low-level background GUS activities (average value of 20 picomoles of 4-methyl umbelliferone/min/mg protein). Individual transformants expressed a range of GUS activities, from levels near those of non-transformed controls to over 1000 picomoles of 4-methyl umbelliferone/min/mg protein.

Genomic DNAs from control and putative transgenic plants were isolated and analyzed for the presence of the *NPT-II* gene (Figure 3). Ten micrograms of genomic DNA from each sample was digested with the indicated restriction enzyme and were hybridized to the 1.0 kb *NPT-II* fragment. Each of the regenerates tested, which had previously been found to demonstrate NPT-II activity, contained sequences that hybridized to the *NPT-II* fragment.

To determine if the hybridizations of the *NPT-II* fragment in tested samples were due to the presence of contaminating residual *Agrobacterium*, genomic DNAs from the above samples were hybridized with the bacterial virulence gene *vir B*. No hybridization of *vir B* was detected in the regenerated plant samples, while a positive control lane containing *Agrobacterium* genomic DNA gave an easily detectable signal (Figure 4).

Example 2

In this Example, meristematic tissue was treated as described in Example 1, with the exception that the tissue was transformed with an *A. tumefaciens* harboring a plasmid having the gene encoding the hepatitis B surface antigen (HBsAG) in lieu of GUS.

The construction of the Ti vector encoding HBsAG (pHB101) was described previously [Mason, *et al.*, *Proc. Natl. Acad. Sci. USA* **89**, 11745-11749 (1992)] and the sequence of the HBsAG gene used in the construct has been published [Pasek, *et al.*, *Nature* **282**, 575-579 (1979)]. *Agrobacterium* strain LBA4404 cells were transformed by the direct method [An, *et al.*, *Methods Enzymol.* **153**, 292-305 (1987)], incorporated by reference herein, with the plasmids prepared from *E. coli* clones, and the structure of the plasmids was verified by restriction digestion. Meristematic tissues were transformed as described in Example 1 by *Agrobacterium* with the plasmid pHB101 (encoding the HBsAG). Plantlets were selectively regenerated on 100 mg/l kanamycin. These putative transformants could be rooted on media containing 100 mg/l of the selective agent, whereas non-transformed controls died under these conditions.

Protein was extracted from leaf tissues by homogenization with a Ten-Broek ground glass homogenizer (clearance, 0.15 mm) in 5 volumes of buffer containing 20 mM sodium phosphate (pH 7.0), 0.15 M NaCl, 20mM sodium ascorbate, 0.1% Triton X-100, and 0.5 mM phenylmethylsulfonyl fluoride at 4 °C. The homogenate was centrifuged at 1000 x *g* for 5 min, and the supernatant was centrifuged at 27,000 x *g* for 15 min. The 27,000 x *g* supernatant was centrifuged at 100,000 x *g* for 1 hr, and the pellet was resuspended in extraction buffer. Protein in the different fractions was measured by the Coomassie dye-binding assay (Bio-Rad). HBsAG was assayed with the Auszyme monoclonal kit (Abbott, North Chicago, IL), using the positive control (HBsAG derived from human serum) as a standard. The positive control was diluted to give HBsAG levels of 0.09-1.8 ng per assay, and the absorbance at 492 nm after color development gave a linear relationship in this range.

Plantlets regenerated on selection media were assayed for the presence of HBsAG. Antigenic positive material was detected in *Musa* plants transformed with pHB101.

Example 3

This is a prophetic example. Meristematic tissue is transformed with an *A. tumefaciens* harboring a plasmid having the gene encoding phosphinothricin acetyltransferase and GUS.

The *bar* gene encodes phosphinothricin acetyltransferase (PAT); which when introduced into transgenic plants gives resistance to the herbicide Basta. The sequence of the *bar* gene used in the construction of the Ti-plasmid encoding PAT was recently published [White, *et al.*, *Nuc. Acids. Res.* **64**, 675-678 (1990)]. A plasmid (pDE110) containing the bar gene under the control of the cauliflower mosaic virus (CaMV) promoter and the nopaline synthase (NOS) terminator is used in the generation of a Ti plasmid (pIBT-115), containing sequences that encode PAT. The *EcoRI/HindIII* fragment containing the CaMV promoter, PAT encoding sequences and NOS terminator is released from pDE110

following an *EcoRI/HindIII* digestion. This fragment is treated with the DNA polymerase Klenow fragment in the presence of excess dNTPs to produce a blunt-ended fragment. PBI21 (CLONETECH Laboratories, Palo Alto, CA), containing the GUS gene under the control of the CaMV promoter, is linearized by digestion with the restriction enzyme *Pst I*. Following digestion, the resulting 3'-OH protrusion is removed from the plasmid by treatment with T4 DNA polymerase in the presence of excess dNTPs at 12 °C. Collectively, these treatments result in a linear plasmid (pBI121) having blunt ends. In addition, this treatment results in the release of a majority of the *NPT-II* gene and its NOS terminator which resides on the PstI/PstI fragment of pBI121. The blunt-ended *EcoRI/HindIII* CaMV-PAT-NOS fragment is ligated into the *NPT-II*-less pBI121 to yield pIBT-115. *Agrobacterium* strain LBA4404 cells are transformed by the direct method [An, *et al.*, *Methods Enzymol.* **153**, 292-305 (1987)] with the plasmids prepared from *E. coli* clones, and the structure of the plasmids is verified by restriction digestion. Meristematic tissues are transformed by *Agrobacterium* with the plasmid pIBT115 (encoding both the *bar* and GUS genes) as described in Example 1, with the exception that phosphinothricin is substituted for kanamycin as the selective agent. Plantlets are selectively regenerated on 0.5 mg/l phosphinothricin. These putative transformants can be rooted on media containing 0.5 mg/l of the herbicide, whereas non-transformed controls die under these conditions. Some of the plants are subsequently grown in soil and tested for herbicide resistance by direct application of the chemical into the leaf whorl when the plants have grown to about a 10 cm height. Severe injury to non-transformed controls is evident in leaf browning while putative transformants show no visible symptoms. It is also observed that herbicide application to control plants cause inhibition of the apical meristem growth with a concomitant proliferation of daughter plants ("suckers"); these affects are not observed in the putative transformants. Since pIBT115 encodes the GUS gene, we also conducted histochemical staining experiments on these plants to score for the presence of GUS. Intense staining was observed in all herbicide resistant plants as compared

to no staining in non-transformed controls under the same conditions of histochemical assay.

Example 4

The transformation of meristems derived as per Example 1 may result in chimeric plants. That is, only one cell in a cluster of cells might receive the *NPT II* DNA, but the resulting enzymatic activity might result in kanamycin degradation that would protect the surrounding cells, thus allowing regeneration of a plant that would contain both transformed and non-transformed cells. To create conditions to eliminate any possible non-transformed cells and cell-derivatives, two additional levels of selection pressure may be used--a rooting regime in the presence of the selection agent, followed by one or more additional rounds of shoot regeneration that force new plant formation from only a small cluster of cells.

For these experiments, 3 x 5 mm shoot tips and 2 - 3 mm thick corm slices were excised from the rooted putative transformant plants recovered on 100 mg/l kanamycin following *Agrobacterium* co-cultivation. These were transferred to shooting medium which also contained the same level of kanamycin. For comparison, non-transformed control meristems or corm slices were transferred to a range of kanamycin concentrations, up to and including 100 mg/l. Inhibition effects in the control plants included stunting of growth and yellowing of leaves; at 100 mg/l, root growth was totally inhibited. A portion of the putative transformants showed vigorous root growth on this level of inhibitor. In a typical experiment, about 40% of the tissues which formed roots on kanamycin formed vigorous roots. These data indicate that the remaining 60% were chimeric, and did not contain meristematic tissues that would give rise to antibiotic-resistant roots. In some cases, following prolonged exposure of putative transformants to kanamycin selection on rooting medium (more than 4 weeks), the death of most of the meristematic shoot tissue was observed in individual samples, with a subsequent onset of new shoot and root development arising from only a small portion of the previously green tissues. We interpret this

phenomenon to be the death on non-transformed tissues, coupled with growth of a new plantlet arising from meristematic tissue derived from an original progenitor cell line which had resulted from an *Agrobacterium*-mediated transformation event.

It was important to determine if the plantlets which had regenerated and rooted on 100 mg/l kanamycin showed evidence of enzymatic activity corresponding to antibiotic degradation. We analyzed multiple individual transformants obtained after *Agrobacterium*-mediated transformation with pBI141, which includes the *NPT II* gene.

Plantlets that formed roots on the selective medium were assayed for enzymatic activity of NPT (Peng *et al., supra*). Approximately 66% of the putative transformants which formed roots on selective media demonstrated easily detectable levels of enzymatic activity.

Individual plants were selected on the basis of these NPT assays for further propagation; the plants which had demonstrated high levels of enzymatic activities were subjected to further meristem culture. This involved dissection of each putative transformant into a small apical meristem and corm slices; these tissues were then transferred to shooting medium containing 100 mg/l kanamycin. Vigorously growing green shoots were subsequently selected, dissected into small (3 x 5 mm) apical meristems and corm slices, and allowed to develop into new shoots which were subsequently forced to root on 100 mg/l kanamycin. From the original 50 plants which were used to start a typical experiment, approximately five vigorously growing putative transformants were obtained after this second round of rooting selection. These were then individually analyzed by further enzymatic and Southern analysis and in all cases, the apical meristem tissue was always preserved and micropropagated to derive multiple clonally propagated derivatives.

Many other variations and modifications may be made in the techniques herein before described, by those having skill in this technology, without departing from the concept of the present invention. Accordingly, it

should be clearly understood that the foregoing description is illustrative only, and not intended as a limitation on the scope of the invention.

CLAIMS

WE CLAIM:

1. A method for transforming a *Musa* plant, said method comprising:

a. wounding meristematic tissue from a *Musa* plant to generate a wounded *Musa* plant tissue and to facilitate access of *Agrobacterium tumefaciens* to *Musa* plant cells competent for transformation and regeneration; and

b. applying to said wounded *Musa* plant tissue at least one transformation competent *Agrobacterium tumefaciens* to transform said *Musa* plant, wherein said at least one transformation competent *Agrobacterium tumefaciens* harbors at least one Ti plasmid and at least one virulence gene, wherein said at least one Ti plasmid comprises at least one genetically engineered T-DNA to effect transformation of said *Musa* plant.

2. The method according to claim 1, wherein the at least one genetically engineered T-DNA comprises at least one first gene selected from the group consisting of genes which code for at least one polypeptide non-native to the *Musa* plant, genes which code for at least one polypeptide native to the *Musa* plant, genes which alter expression of at least one native *Musa* gene, and combinations thereof.

3. The method according to claim 2, wherein the at least one genetically engineered T-DNA further comprises at least one second gene selected from the group consisting of genes which code for selection agent resistance, genes which code for at least one screenable marker, and combinations thereof.

4. The method according to claim 2, wherein the at least one genetically engineered T-DNA further comprises at least one gene which codes for selection agent resistance, and wherein the method further comprises regenerating the transformed *Musa* plant tissue in the presence of at least one selection agent

responsive to the at least one gene which codes for selection agent resistance so as to select for resistant tissue transformed with the at least one genetically engineered T-DNA.

5. The method according to claim 1, wherein the *Musa* plant is selected from the group consisting of banana and plantain.

6. The method according to claim 1, wherein the meristematic tissue is selected from the group consisting of apical meristem, adventitious meristem, and combinations thereof.

7. The method according to claim 1, wherein the wounding is by dissection.

8. The method according to claim 1, further comprising bombarding the meristematic tissue with microparticles prior to applying the at least one transformation competent *Agrobacterium tumefaciens*, but after wounding the meristematic tissue.

9. The method according to claim 1, further comprising co-cultivating the wounded *Musa* plant tissue with *Agrobacterium tumefaciens* and at least one compound for inducing the at least one virulence gene of *Agrobacterium tumefaciens*.

10. The method according to claim 9, wherein the at least one compound for inducing the at least one virulence gene of *Agrobacterium tumefaciens* is acetosyringone.

11. The method according to claim 2, wherein the at least one first gene which codes for at least one polypeptide non-native to the *Musa* plant codes for resistance to at least one herbicide.

12. The method according to claim 11, wherein the at least one herbicide is selected from the group consisting of phosphinothricin, glyphosate, sulfonylureas, and combinations thereof.

13. The method according to claim 2, wherein the at least one first gene which codes for at least one polypeptide non-native to the *Musa* plant codes for at least one pharmaceutical.

14. The method according to claim 13, wherein the at least one pharmaceutical is selected from the group consisting of hepatitis B surface antigen, Norwalk virus capsid protein, insulin, interleukins, growth hormone, erythropoietin, G-CSF, GM-CSF, hPG-CSF, M-CSF, Factor VIII, Factor IX, tPA, insulin, and combinations thereof.

15. The method according to claim 3, wherein the at least one second gene which codes for at least one screenable marker is selected from the group consisting of GUS, CAT, and combinations thereof.

16. The method according to claim 3, wherein the at least one second gene which codes for selection agent resistance codes for antibiotic resistance.

17. The method according to claim 16, wherein the at least one second gene which codes for antibiotic resistance codes for resistance to at least one antibiotic selected from the group consisting of kanamycin, hygromycin, and combinations thereof.

18. The method according to claim 3, wherein the at least one second gene which codes for selection agent resistance codes for herbicide resistance.

19. The method according to claim 18, wherein the at least one second gene which codes for herbicide resistance codes for resistance to at least one herbicide selected from the group consisting of phosphinothricin, glyphosate, sulfonylureas, and combinations thereof.

20. The method according to claim 1, wherein the at least one genetically engineered T-DNA codes for at least one industrial enzyme.

21. The method according to claim 1, wherein the at least one genetically engineered T-DNA codes for at least one fusion protein.

22. The method according to claim 1, wherein the at least one genetically engineered T-DNA codes for at least one protein that interacts with at least one compound in the *Musa* plant to produce a secondary metabolite of said at least one compound.

23. The method according to claim 1, wherein the at least one genetically engineered T-DNA codes for at least one protein that changes at least one phenotypic trait of the fruit of the *Musa* plant.

24. The method according to claim 23, wherein at least one phenotypic trait is selected from the group consisting of texture, flavor, pigmentation, and combinations thereof.

25. The method according to claim 23, wherein the at least one phenotypic trait affects the ripening of the fruit of the *Musa* plant.

26. A *Musa* plant produced by the method of claim 1, which includes cells which comprise in their genome at least one gene selected from the group consisting of at least one gene which codes for at least one polypeptide non-native

to the *Musa* plant, at least one gene which codes for at least one polypeptide native to the *Musa* plant, at least one gene which codes for altered gene expression of at least one native *Musa* gene, and combinations thereof.

27. A method according to claim 1, further comprising:

a) growing said transformed *Musa* plant for a sufficient time to identify the presence of chimeric features;

b) producing nonchimeric tissue by dividing said transformed *Musa* plant into segments which have at least one meristem which can regenerate into an intact plant and which have cells that are uniformly transformed to produce nonchimeric tissue; and

c) growing said nonchimeric tissue into a nonchimeric plant.

28. A transformed plant made by the method of claim 1.

29. Vegetatively-derived progeny of the transformed plant of claim 26.

30. Plant parts obtained from the transformed plant of claim 26.

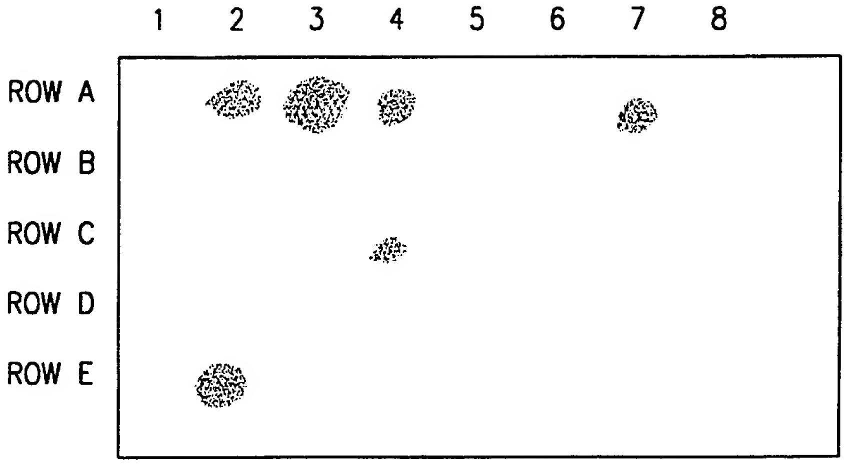
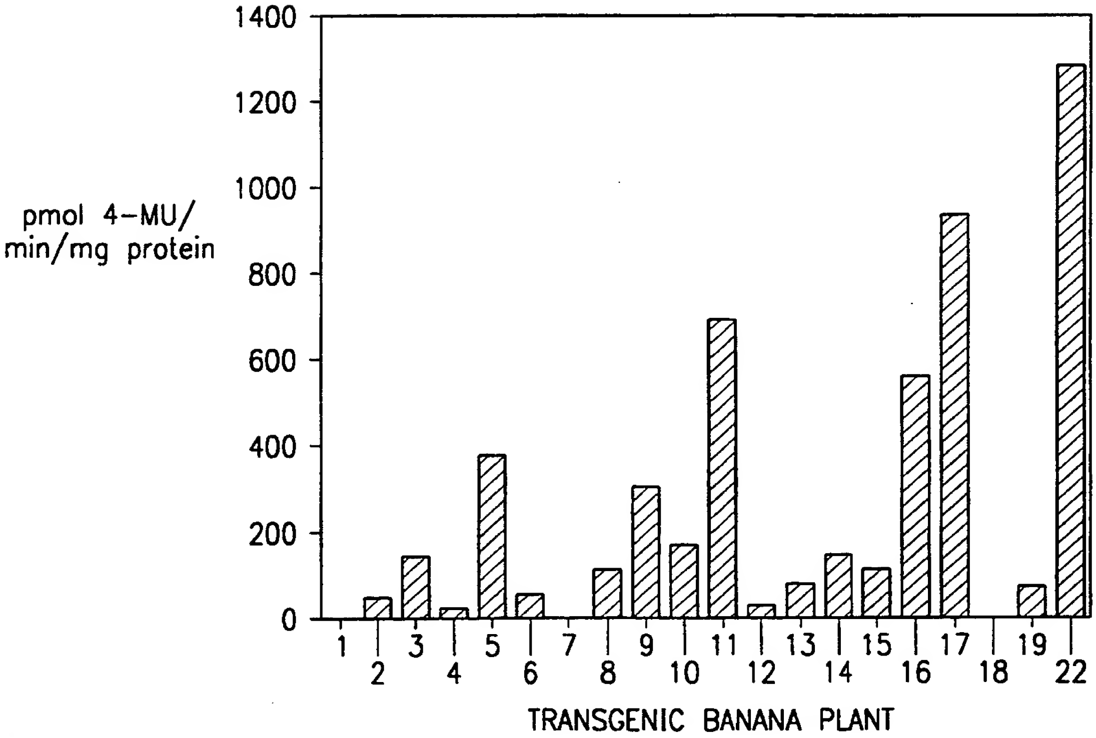

1
2
3
4
5
6
7
8
ROW A
ROW B
ROW C
ROW D
ROW E

FIG. 1


1400
1200
1000
800
600
400
200
0
pmol 4-MU/
min/mg protein
1
2
3
4
5
6
7
8
9
10
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13
14
15
16
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18
19
22
TRANSGENIC BANANA PLANT

FIG. 2

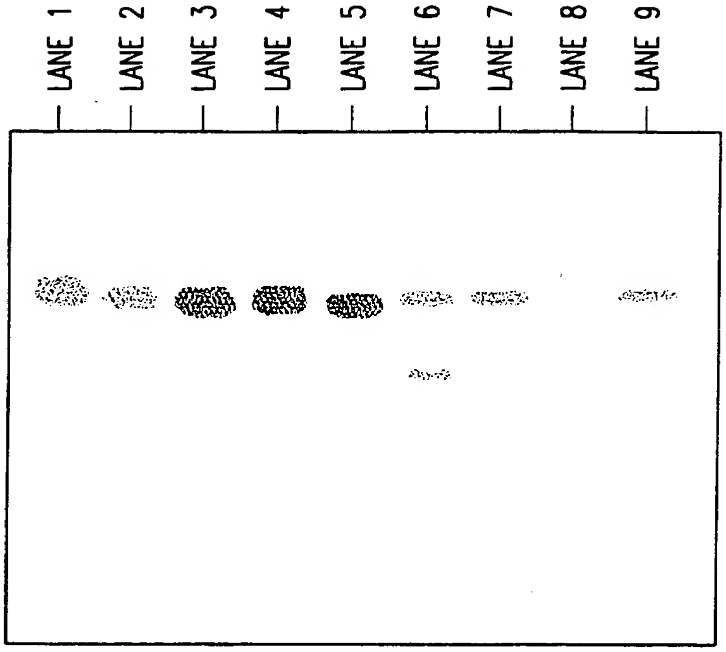
LANE 1
LANE 2
LANE 3
LANE 4
LANE 5
LANE 6
LANE 7
LANE 8
LANE 9

FIG. 3

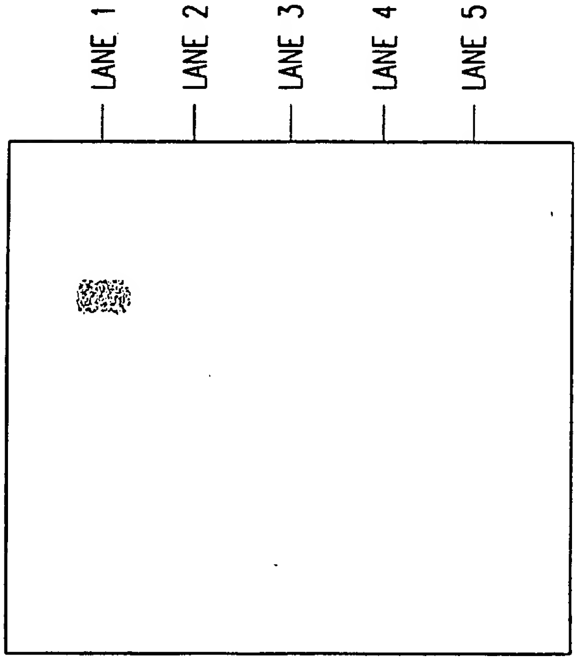
LANE 1
LANE 2
LANE 3
LANE 4
LANE 5

FIG. 4

INT[illegible]ATIONAL SEARCH REPORT

International application No.
PCT/US94/14210

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A01H 5/00, 5/08; C12N 5/14, 15/64, 15/82
US CL :435/69.1, 69.3, 69.4, 69.52, 70.1, 172.3; 800/205
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 69.3, 69.4, 69.52, 70.1, 172.3; 800/205

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,956,282 (GOODMAN ET AL) 11 SEPTEMBER 1990, see column 2, lines 10-22, column 3, lines 11-30.	13-14
Y	PLANT PHYSIOLOGY, Volume 95, issued 1991, J. Gould et al, "Transformation of *Zea mays* L. Using *Agrobacterium tumefaciens* and the Shoot Apex", pages 426-434, see pages 427 and 428.	1-30
Y	THE PLANT CELL, Volume 2, issued July 1990, W. J. Gordon-Kamm et al, "Transformation of Maize Cells and Regeneration of Fertile Transgenic Plants", pages 603-618, see page 604.	1-30

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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Date of the actual completion of the international search
06 FEBRUARY 1995

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INTERNAT[illegible]AL SEARCH REPORT

[illegible]national application No.
[illegible]CT/US94/14210

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BIO/TECHNOLOGY, Volume 7, No. 2, issued February 1989, F. J. Novak et al, "Somatic Embryogenesis and Plant Regeneration in Suspension Cultures of Dessert (AA and AAA) and Cooking (ABB) Bananas (Musa spp.)", pages 154-159, see page 156.	1-30
Y	W. R. SHARP et al, "HANDBOOK OF PLANT CELL CULTURE, VOLUME 2", published 1984 by Macmillan Publishing Company (N.Y.), pages 327-348, see pages 338-339.	1-30

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International Bureau

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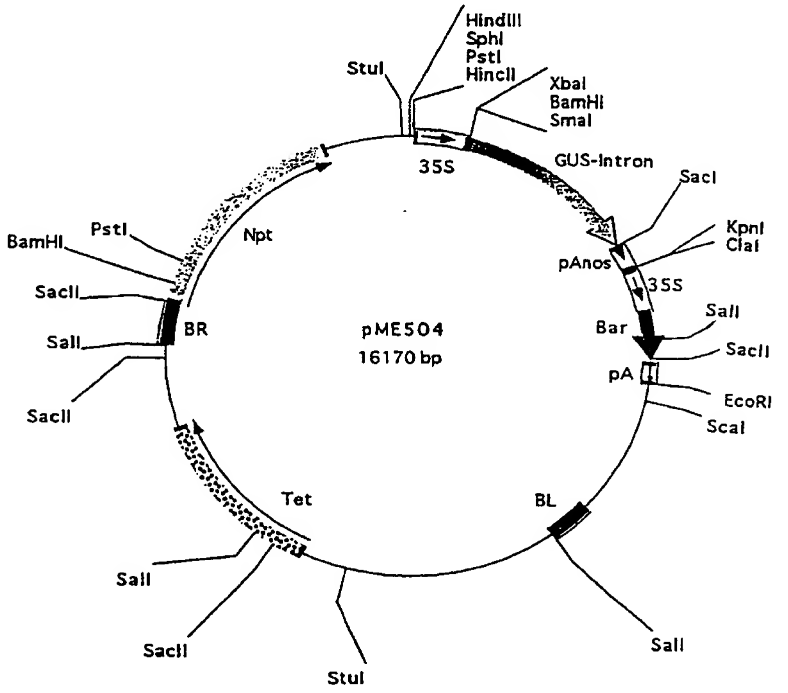
(54) Title: TRANSGENIC LEMNACEAE

(57) Abstract

The present invention concerns genetically stable transformed *Lemnaceae* plants and methods for their transformation by *Agrobacterium* cells. The present invention further concerns a method for regeneration of plants from *calli*, utilizing low sucrose media and products of interest produce from said plants. The present invention further concerns booster media for use in the above methods.



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TRANSGENIC LEMNACEAE

FIELD OF THE INVENTION

The present invention relates to stably transformed plants, progeny thereof and products obtained from the cells or progeny. The invention further concerns methods for the genetic transformation of plants and more specifically to a method wherein *Agrobacterium* is used as the transforming vector.

PRIOR ART

The prior art considered to be pertinent to the following disclosure is listed in the section entitled *"References"* before the claims.

BACKGROUND OF THE INVENTION

Genetic transformation of plants is gradually beginning to play an important role in modern agriculture. Attempts are made to introduce heterologous DNA into plants in order to increase their resistance to viral infection, acquire or increase resistance to various herbicides, modulate ripening or decay times, increase the nutritional value of various plant products, bring them to produce pharmaceuticals, and produce various other chemical and biological molecules.

Commercial production of transgenic compounds in bacterial, yeast and mammalian cell systems is often beset by high capital investment costs in fermentation equipment and the necessity to eliminate prion or microplasmal components from the purified product. Recently, production of heterologous proteins and peptides (e.g., α-amylase, antibodies, enkephalins, human serum albumin) has been achieved in plants (Pen *et al.*, 1992, Miele, 1997). Potential advantages of transgenic plant systems are: lowered production costs of biomass and a reduction in the biohazard of contaminants in downstream processing of the products. Transgenic plants may thus be superior bioreactors for bulk enzymes in industry, purified products in medicine and orally active pharmaceuticals.

In order to transform plants to produce a desired product, the relevant gene, once identified and cloned, has to be introduced into the plant of interest so that the resulting plant is capable of passing the gene to its progeny. The methods

of introduction proposed for this purpose include electroporation, microinjection, microprojectile bombardments, liposome fusion, *Agrobacterium* mediated transfer, and many others.

One of the most commonly used transforming vectors is *Agrobacterium*, which is a genus of plant pathogenic bacteria of the family *Rhizobiaceae*, which does not fix free nitrogen and usually produces gall and hairy roots in infected cells. Heterologous DNA is introduced into the *Agrobacterium* and through a process of transfection wherein genetic material from the *Agrobacterium* enters the plant's cell, genetic transformation of the plant takes place (Armitage *et al.*, 1992). *Agrobacterium* infects primarily dicotyledonous plants and infects monocotyledonous plants only at a very low yield (Armitage *et al.*, 1992).

One attempt to transform monocotyledonous plants was by a particle gun wherein the heterologous DNA is delivered by air or by helium into the plant or plant cell to be transformed. This technique has two main disadvantages: first, it is quite difficult to target the DNA particles to the meristematic zone wherein, for certain plants such as those of the family *Lemnaceae*, the transformation should take place in order to enable regeneration therefrom of a full transformed plant; second, even if the DNA particle enters the cell in the meristematic zone and reaches the nucleus thereof, the DNA does not usually integrate into the cell's chromosome and, thus after a few cell cycles the unintegrated heterologous DNA is lost, so that transformation by a particle gun is usually merely transient.

It would have been highly desirable to provide a method for the genetic transformation of monocotyledonous plants which would result in stable transformation with a satisfactory yield.

One of the most commercially promising monocotyledons are the *Lemnaceae*, a widely distributed aquatic family of small (1-5 mm) plants. The *Lemnaceae* excel in two characteristics potentially exploitable by the biotechnology industry: their extraordinary vegetative growth rates and a high tolerance for a spectrum of nutrients and toxic substances (Landolt and Kandeler, 1987). In the U.S.A., commercialization of *Lemnaceae* has centered around waste water management and animal feed (Culley *et al.*, 1981; Ngo, 1987). However, the use of mixed aquacultures and conventional technology has met with only moderate

success. A different approach was taken in Israel, utilizing the *Lemna gibba Hurfeish* strain (Porath *et al.*, 1979). With its especially short root and high protein, carotenoid and iron content, this strain was cultivated under modern greenhouse conditions (4 tons harvested per acre per week; Tzora Biotechnology Inc., Kibbutz Tzora), and successfully marketed as a packaged vegetable product for the food industry. Notwithstanding the exceedingly high growth rates and the promising future of *Lemnaceae* as a potential food source, various attempts to genetically transform these plants, by a stable transformation method proved, to date, quite unsuccessful. The failure of transformation was due to the fact that *Lemnaceae* multiply vegetatively, daughter fronts arising from meristematic zones deep inside the mother frond. Thus, the meristem initial must be reached for stable transformation to take hold. Particle bombardment of *Lemnaceae,* the current state-of-the-art method used by several groups to obtain localized, transitory transformation events, was found by the inventors of the application ineffective in transformation of daughter fronds.

It would have been highly desirable to obtain *Lemnaceae* plants which are stably transformed with heterologous DNA of interest and to use such transformed plants for the production of chemical and biological products.

GENERAL DESCRIPTION OF THE INVENTION

In the following description, the term *"transformation"* will be used to denote the introduction of a transforming DNA into plant cells or tissues which brings to the appearance in these cells or tissues of traits which said cells or tissues did not possess beforehand or to modulation of traits present, *a priori*, in the plants. The term *"stable transformation"* will be used to denote such a genetic transformation which is heritable to future generations of the transformed plant. The term *"transforming DNA"* will be used herein to denote a foreign DNA molecule which is introduced into plant cells and causes their transformation. The transforming DNA may be of any origin, for example plant origin, and may also be a DNA sequence which is naturally present in the transformed plant. The transforming DNA may comprise coding sequences and/or control sequences capable of regulating the amount and time of the transcription. The term "*stably*

transformed plant" will be used hereinafter to denote a plant comprising a transforming DNA stably integrated in its genome. A *"stably transformed Lemnaceae"* conforms to the description of a stably transformed plant.

In accordance with the present invention it was surprisingly found that there exist conditions which allow stable transformation of *Lemnaceae* plants. Thus, by one of its aspects, the present invention concerns a stably transformed *Lemnaceae* plant, tissues, products thereof and progeny thereof.

The *Lemnaceae* plants are preferably of the genera: *Spirodela, Lemna* and *Wolffia*. The present invention preferably concerns transformed *Lemnaceae* strains capable of exceptionally high efficiency of transformation, an example of such a strain being *Spirodela punctata* strain 8717, which is a *Spirodela punctata* strain isolated by E. Landolt and erroneously labeled as *Lemna disperma* in Landolt 1986.

The transformed *Lemnaceae* plant, tissue and products thereof of the invention may be used for the production of various chemical and biological products such as proteins and polypeptides encoded by the transforming DNA and may also be used to prepare various enzymes capable of producing various chemicals such as carbohydrates, lipids, alkaloids, pigments, vitamins, etc.

The present invention also concerns a method of production of a product of interest, for example chemical and biological products such as proteins, polypeptides, carbohydrates, lipids, alkaloids, pigments, vitamins, and others, wherein a transformed *Lemnaceae* according to the invntion is grown in an appropriate culture medium, to produce the product of interest. The product of interest may be further isolated and purified, totally or partially, for a furtehr use, in order to serve as a food additive, a cosmetic additive, a vaccine, therapeutic agent, a biocatalyst for enzymatic conversion of chemicals, etc. Alternatively, the product of interest may be used in its raw, unisolated form as present in the grown *Lemnaceae* plant, by using the plant with no or partial processing itself for the above purposes.

The present invention is also concerned, by another of its aspects, with a product of interest being a chemical or biological product such as proteins, polypeptides, carbohydrates, lipids, alkaloids, pigments, vitamins, and others,

obtained from the above stably transformed *Lemnaceae* plants.

The transformed plant or tissue may also express desired traits which are not featured in production of new products, examples of such traits are: antibiotic resistance, for example, kanamycin resistance, conferred by the *npt II* gene; or herbicide resistance, for example, resistance to the herbicited BASTA 20 (ammonium glufosinate, Hoechst, Germany)

The transferred *Lemnaceae* plants or tissue may also express more than one foreign gene, for example, the plant may be transformed to be resistant to several herbicides and/or antibiotics at once.

In accordance with the present invention, it was found that stable transformation of *Lemnaceae* plants or tissue may be obtained by the use of *Agrobacterium* cells carrying said transforming DNA. Thus, in accordance with a further of its aspects, the present invention concerns a method for the stable transformation of *Lemnaceae* plants or tissue which comprises incubating *Lemnaceae* plants or plant tissue with *Agrobacterium* cells carrying said transforming DNA, whereby cells in said plant tissue become stably transformed by said transforming DNA.

It was further found that there exists *Agrobacterium* strains which can specifically target and transform meristematic tissue in *Lemnaceae*, for example *A. tumefaciens* strains EHA105, EHA101 and GVE3103, or *Agrobacterium* strains which can specifically target and transform the wounded area of the plant such as *A. tumefaciens* strains LBA4404 and C58. Therefore the method of the invention preferably concerns incubation of *Lemnaceae* plants with *Agrobacterium* of the strains. EHA105, EHA101 and GVE3103 capable of transforming the meristematic tissue or *Agrobacterium* strains LBA4404 and C58 capable of transforming wounded tissue.

It was still further found that use of vacuum filtration during the incubation of the *Lemnaceae* plants with the *Agrobacterium* cells increases the efficiency of transformation. Thus, by a preferred embodiment, the method of transformation includes incubation of *Lemnaceae* plants or tissue with *Agrobacterium* cells while applying vacuum infiltration.

Another embodiment of the method of the invention is based on the

finding that it was possible to increase the efficiency of *Lemnaceae* transformation by *Agrobacterium* by exposing the meristematic zone of the mother frond. Such exposure can be carried out physically, i.e. by removing the daughter frond to expose the meristematic zone, for example, by a plucking motion using forceps, or by any other mechanical means. Alternatively, said exposure may be carried out by applying chemical preparation or a hormone preparation capable of specifically removing the daughter found without damaging the underlying meristematic zone.

Yet another aspect of the present invention concerns a novel method for plant transformation using transforming *Agrobacterium* cells, which is particularly suitable for mass transformation of plant tissue. In accordance with this method, plants are cut into small particles which are then incubated with the transforming *Agrobacterium* cells, preferably in the presence of the booster medium which will be described hereinbelow. The size of the particles should be such that at least some of them will contain undamaged meristematic tissue which is capable of regenerating into full plants. In order to achieve this feature, the particles should preferably be at an average size of above 150 μm in diameter, most preferably at a size range of about 150 μm - 750 μm. Cutting the plant tissue into such small particles maximizes the contact area between the meristematic tissue and the *Agrobacterium*. Furthermore, *Agrobacterium* cells more readily infect damaged plant tissue and by cutting the plant, the *Agrobacterium* cells are exposed to large regions of damaged plant tissue. The overall result of these factors is a marked increase in the transformation yield.

Another transformation method which may be used in the performance of the present invention, is microinjection which is known *per se*. In accordance with this method, *Agrobacterium* cells, preferably together with the booster medium of the invention which will be described hereinbelow, are microinjected to a desired zone of transformation within the plant, typically into the plant's meristem. One major advantage of microinjection, is that it allows specific targeting of the transforming *Agrobacterium* cells to a desired tissue, e.g. only to the roots' meristem, only to the leaves' meristem, etc., so that the result is a plant having foreign DNA only at a specific tissue, for example, the roots and not in other tissues.

Another embodiment of the method of transformation of *Lemnaceae* is based on the surprising finding that transformation can be carried out *in planta*, i.e. utilizing the full plant and there is no need to cut the plant to small particles, or to use tissue culture and then *in vitro* regeneration for transformation purposes. A full plant can be used for transformation provided that the *Agrobacterium* cells are targeted to the meristem either by direct microinjection as described above or by utilization of *Agrobacterium* strains which preferably target the meristem such as *A. tumefaciens* strain EHA105, EHA101 and GVE3103. Thus the present invention provides a method for *in planta* transformation of *Lemnaceae* by targeting *Agrobacterium* cells carrying the transforming DNA to the meristem of the plant to be transformed.

In accordance with another embodiment of the invention, it was found that it is possible to increase the efficiency of transformation of plants by *Agrobacterium* cells by incubating the *Agrobacterium* cells with the plant tissue to be transformed in the presence of a booster medium which is capable of increasing the *Agrobacterium's* virulence. This increase in efficiency due to use of the booster is not limited to the transformation of *Lemnaceae* plants but is also applicable to plants in general including monocotyledonous plants and dicotyledonous plants.

Agrobacterium is already routinely used for transformation of dicotyledonous plants. However, in accordance with this embodiment of the invention, the efficiency of transformation of dicotyledonous plants is increased by incubation of the *Agrobacterium* booster medium. With respect to monocotyledonous plants, although there have been some reports of a few successful transformations of such plants by *Agrobacterium*, these reports have been sporadic and usually showed unsatisfactory transformation yields. Increasing *Agrobacterium's* virulence by the booster medium, in accordance with said embodiment, allows for the first time, the transformation of many species of monocotyledonous plants which were previously untransformed, including those belonging to the genus *Lemnaceae*, as well as an increase in the yield of transformation of plants already known to be transformed, albeit at a low yield by the use of *Agrobacterium*.

Stably transformed plants produced by utilizing the booster medium as described above, also form an aspect of the invention.

The booster medium which enhances the virulence of the *Agrobacterium* cells, comprises plant tissue cultured at a pH below about 5.2. For example, the booster medium may comprise a fresh cell suspension of dicotyledonous plants, at a concentration of 1-10% (w/v). The fresh cell suspension may be, for example, from dicotyledonous plants of the *Solanaceae* family.

Preferably, the booster medium also comprises caffeine at a concentration of 100-500 mg per liter of medium.

A specific example of a booster medium is one comprising MS basal medium at a pH of about 3.5 - 4.2, 1-10% (w/v) of a fresh cell suspension of *Nicotiana tabacum*, and about 100-500 mg per liter of medium caffeine.

By another alternative, the booster medium of the invention is a plant growth medium comprising *Lemnaceae* plant extracts. Such a medium can be produced by extracting *Lemnaceae* plants in a suitable medium such as phosphate buffer.

Both types of booster mediums, having either or both of the above specifications for use in enhancing transformation efficiency of *Agrobacterium* cells used as a transformation vector, also form another aspect of the invention.

By yet a further embodiment, the present invention concerns a method for maintaining morphogenetic *Lemnaceae* calli for long periods of time, by using low levels of sucrose in the growth medium, for example, from 0.1 to 1.5% sucrose. It was found that it is possible to increase significantly the period of maintaining calli in a viable state, for example, from 2 weeks to more than 3 months by decreasing the sucrose level in the growth medium.

Another aspect of the invention concerns a method for the production of highly regenerative *Lemnaceae* calli, and furthermore a method for rapid and efficient regeneration of the plants from the calli, utilizing the combined effect of B5 minerals, low sucrose levels (0.1 to 1.5% sucrose) and phytohormones in the growth medium.

The present invention will now be illustrated with reference to some non-limiting examples.

BRIEF DESCRIPTION OF THE DRAWING

Fig. 1 shows a schematic picture of plasmid Ti pME504.

DETAILED DESCRIPTION OF THE INVENTION

In the following, the invention will be illustrated at times with specific reference to the transformation of *Lemnaceae* plants, tissue or callus, such reference being given merely as an example and it should be understood that the invention is not limited thereto.

The stably transformed *Lemnaceae* plants contain foreign genes which confer useful traits such as: improvement of nutritional quality of the plant or plant parts; *de novo* expression of desired chemical and biological products, e.g. enzymes; growth factors, hormones such as insulin; antibodies; anti-oxidants; defensins; proanthocyanidins; cytokines and other biologically active polypeptides and proteins; over-expression of products already expressed by these plants; etc. Other products that may be obtained from the transformed *Lemnaceae* plants are enzymes for industrial applications, such as super-oxide dismutase (SOD), α-amylase, invertase, sucrose phosphate synthase and the like, and chemicals such as food pigments, e.g. β-carotene, anthocyanin, etc. The type of product obtainable from the transformed plants is obviously contingent on the nature of said transforming DNA. By another application the genes may be those which impart disease resistance.

Genes coding for proteins imparting disease resistance are known in the art, including lytic peptides, defensins, oxalate oxydase genes for tolerance to sclerotinia or chitinases (US 5,597,946, US 4,940,840, US 5,290,687, US 5,374,540, US 5,670,706, US 5,399,6801, US 5,695,939, all publications incorporated herein by reference).

The transforming DNA which is introduced into the plant cells, include, as will be appreciated by the artisan, coding sequences which code for the desired trait as well as control sequences which control expression of the coding sequence, for example, promotors, enhancers, terminaters, introns and the like, pre-pro peptides or transit peptides, the latter driving the expression of said desired trait in

a specific targeted region of the plant cell.

Promoters controlling the expression of genes in plant cells are well known in the field of plant biotechnology, including any promoter sequence of a gene naturally expressed in plants or plant cells, form plant, viral or bacterial origin. Suitable promoters are disclosed in Weising et al. (1988), *Annual Rev. Genet.*, **22**: 241), the subject matter of which is incorporated herein by reference. The following is a partial representative list of promoters suitable for use in the context of the invention: regulatory sequences from the T-DNA of *A. tumefaciens*, including mannopine synthase, nopaline synthase and octopine synthase; regulatory sequences from plant origin, including alcohol dehydrogenase promoter from corn, light inducible promoters such as ribulose-biscarboxylase/oxygenase small subunit promoters (SSU RuBisCO) from genes of a variety of species and the major chlorophyl a/b binding gene promoters, histone promoters (EP 507 698), actin promoters (US 5,641,876), maize ubiquitin 1 promoters (Christenses *et al.*, (1996)), regulatory sequences from viral origins, such as 19S or 35S promoters of the cauliflower mosaic virus, (US 5,352,605; US 5,530,196); developmentally regulated promoters such as waxy, zein, or bronze promoters from maize; as well as synthetic or other natural promoters which are either inducible or constitutive, including those promoters exhibiting organ specific expression or expression at specific development stage(s) of the plant, like the promoter of napin (EP 255 378) or the alpha-tubulin promoter (US 5,635,618); all publications being incorported herein by reference.

As a preferred embodiment, the promoter is selected among the group consisting in the ribulose-biscarboxylase/oxygenase small subunit promoters (SSU RuBisCO) from genes of a variety of species, the histone promoters, the actin promoters, the maize ubiquitin 1 promoters and the 35S promoters of the cauliflower mosaic virus (CaMV 35S).

According to the present invention, it is possible to use with the promoter, other regulatory elements usually located between the promoter and the coding sequence of the desired trait, which elements induce the expression of the said desired trait in a specific target region of the plant or plant cell, for example, the chloroplasts. Examples are coding sequences for transit peptides, single or

combined multiple sequences, the latter may be separated by intermediate sequences. Such multiple transit peptide sequences, such as double transit peptide sequences, may comprise, in the direction of transcription (5' to 3'): a transit peptide of a plant gene encoding a plastid-localized enzyme, a partial sequence of the N-terminal mature part of a plant gene encoding a plastid-localized enzyme and then a second transit peptide of a plant gene encoding a plastid-localized enzyme. An example is the optimized transit peptide disclosed in US 5,510,471 or US 5,633,448 (incorporated herein by reference). The plastid-localized enzymes may be of any origin, for example the small subunit (SSU) of the ribulose, 1,5-diphosphate carboxylase oxygenase (RuBisCO) gene,or the plant EPSPS gene. The signal peptide of the tobacco PR-1a gene described in Cornelissen *et al.* is another example of a transit peptide.

Another control region may be a terminator or untranslated polyadenylation signal region at the 3' terminus of the coding sequene which may be of any origin, for example bacterial, such as the nopaline synthase gene of *Agrobacterium tumefaciens*, or of plant origin, such as the terminator of the gene coding for the SSU RuBisCO of maize or sunflower, or the terminator of a plant histone gene such as disclosed in EP 633,317, incorporated herein by reference.

Furthermore, the transforming DNA may comprise also a selectable marker gene, such as a gene coding for herbicidal resistance, resistance to antibiotics, or the like. In addition or in the alternative, the transforming DNA may further comprise a reporter gene, such as a gene coding for a color marker. A selectable marker gene or a reporter gene facilitates identification and selection of the transformed tissue and enables its separation from untransformed tissue.

Specific examples of selectable marker genes are the hygromycin phosphotransferase (HPT) coding sequence, which may be derived from *E.coli* and which confers resistance to the antibiotic hygromycin B; the aminoglycoside phospho-transferase gene of transposon Tn5 (AphII) which encodes resistance to the antibiotics kanamycin; neomycin and G418. Genes coding for protein imparting herbicide tolerance are known in the art, including genes imparting tolerance to oxynil herbicides (US 4,810,648 and US 5,559,024), genes imparting tolerance to glyphosate and EPSPS inhibitor herbicides (US 4,535,060, US 4,769,061, US

5,094,945, US 4,940,835, US 5,188,642, US 4,971,908, US 5,145,783, US 5,312,910, US 5,310,667, US 5,633,435, US 5,627,061, US 5,554,798, US 5,633,448, WO 96/04103, all publications incorporated herein by reference), genes imparting tolerance to glufosinate (EP 242 236, incorporated herein by reference), as well as genes imparting tolerance to HPPD inhibitors (WO 96/38567 and WO 98/02562, both publications incorporated herein by reference). Those selectable marker genes which confer resistance or tolerance to these phytotoxic compounds are also of commercial utility in the resulting transformed plants.

Reporter genes may be used for identifying transformed cells, tissue or calli and for evaluating the functionality of regulatory sequences. Reporter genes which code for easily assayable selectable marker proteins are well known in the art. In general, a reporter gene is a gene which is not present in, or expressed by, the recipient organism or tissue and which codes a protein which expression is manifested by some easily detectable property, e.g., phenotypic change or enzymatic activity. Examples of such genes are the chloramphenicol acetyl transferase gene (*CAT*) from Tn9 of *E. coli*, the β-glucuronidase gene (*GUS*) of the *uidA* locus of *E. coli*, the green fluorescence protein (GFP) obtained from *A. Victoria* and the luciferase gene from the firefly *Photinus pyralis*. Expression of the reporter gene is assayed at a suitable time after the DNA has been introduced into the recipient cells. An example of such an assay entails the use of the *E. coli* β-glucuronidase (*GUS*) gene (Jefferson *et al.*, (1987)). Plant cells transformed and expressing this gene will stain blue upon exposure to the substrate, 5-bromo-4-chloro-3-idolyl-β-D-glucuronide (X-GLUC), in the extracellular medium.

According to the method of the invention, *Agrobacterium*, e.g. *Agrobacterium tumefaciens*, is engineered so as to contain the transforming DNA to be inserted into the target plant, e.g. the target *Lemnaceae* plant, which engineering is performed by means known *per se*. The whole plant or the plant cells tissue or callus are then brought into contact with the *Agrobacterium* cells and incubated together. The plant tissues are then selected for those containing the transforming DNA, for example, by testing for phenotypic expression of the marker gene, e.g. herbicidal or antibiotic resistance, or for the expression of the reporter gene, e.g. a color product. It is also possible to verify the presence of the

introduced transforming DNA by a DNA assay such as by PCR.

The invention will now be illustrated further in the following examples:

EXPERIMENTAL PROCEDURES

I. Culture and maintenance of *Lemna* and *Spirodela* for microinjection experiments

For meristem-zone microinjection, an axenic inoculum (approx. 10 plants) of *Spirodela oligorrhiza* (herein called *Spirodela punctata*) Hegelm. or *Lemna gibba* Hurfeish was introduced in a 250 ml flask containing 50 ml of MS medium having the ingredients as detailed in the following Table 1.

II. Standard growth conditions

Cultures were grown at 26°C under continuous fluorescent light (30 μE m^2s^{-1}) in a 3-5% CO_2-enriched atmosphere.

Table 1:

Modified MS basal medium (MS medium) (based on Murashige & Skoog, 1962)

Macro elements	Amount (mg/l)	Micro elements	Amount (mg/l)	Organic additives	Amount (mg/l)
NH_4NO_3	1650	H_3BO_3	6.2	Glycine	2
KNO_3	1900	$MnSO_4$	22.3	Meso-inositol	100
$CaCl_2 \cdot 2H_2O$	440	$ZnSO_4 \cdot 2H_2O$	0.25	Thiamine HCl	10
$MgSO_4 \cdot 7H_2O$	370	KI	0.83	Nicotinic acid	0.5
FeEDTA	35	$Na_2MoO_4 \cdot 2H_2O$	0.25	Pyridoxine	0.5
KH_2PO_4	170	$CuSO_4 \cdot 5H_2O$	0.25	Biotin	0.5
		$CoSO_4 \cdot 7H_2O$	0.03	Folic acid	0.5
				Casein hydrolysate	800
				Sucrose	30000
				pH brought to 5.8 with NaOH prior to autoclaving	

III. Standard Transformation Procedure

Under sterile conditions, 2.5 g *Lemnaceae* plants were placed in an

empty 9 cm Petri dish. A 10 ml suspension of 5-8x10^8/ml *A. tumefaciens*, in MS Basal medium (Table 1) was prepared as described in (IV) below, and added to the dish. Plants were co-cultivated with the *A. tumefaciens* suspension for 20-40 min. at room temperature. The suspension was removed from the dish and the plants washed 3 times with fresh MS Basal medium (Table 1). The plants were transferred to a vessel (15x20x12cm) containing 1.5 l SP medium (Table 2) at 26°C under continuous fluorescent light (30 μE m^2s^{-1}) in a 3-5% CO_2-enriched atmosphere.

Table 2

SP medium (modified from Hutner) (cf., Posner, 1967)

	Amount (mg/l)
KNO_3	300
$Ca(NO_3)_2 \cdot 4H_2O$	72
$MgSO_4 \cdot 7H_2O$	74
KH_2PO_4	40
NaEDTA	0.003
Ferric citrate	1
H_3BO_3	1
$MnSO_4$	0.1
$Na_2MoO_4 \cdot 2H_2O$	0.1
$CuSO_4 \cdot 5H_2O$	0.03
$Z_nSO_4 \cdot 4H_2O$	1
pH brought to 5.8 with NaOH prior to autoclaving	

IV. Preparation of *A. tumefaciens* for *Lemnaceae* transformation

A single colony of *Agrobacterium tumefaciens*, maintained on antibiotic-supplemented LB plates (Suppl. LB medium;), (Table 3, below), was picked and grown overnight (28°C, 250 rpm) in 10 ml of antibiotic-supplemented 2YT broth (Suppl. 2YT 1 medium;) (Table 4 below). The grown culture was transferred to 50 ml of Suppl. 2YT medium and further grown for an additional 12 hr (28°C, 250 rpm). Before transformation, the *A. tumefaciens* culture was centrifuge (3200 x g, 5 min.), the supernatant discarded and the bacteria resuspended in 10 ml of MS medium (Table 1). Before co-cultivation with *Lemnaceae* plants, the bacterial concentration was adjusted to 5-8x10^8 cells/ml.

Table 3

Supplemented and solidified liquid broth medium (Suppl. LB medium)

	Amount
Bacto tryptone	10 g
Bacto yeast extract	5 g
NaCl	10 g
Rifampicin	25 mg
Kanamycin	50 mg
Carbenicillin	50 mg
Cifco agar	10 g
pH brought to 7.0 with NaOH	

V. Meristem-zone microinjection

1. Preparation of *Lemnaceae* plants - Axenic *Lemnaceae* plants were cultured in containers (8.5 cm diameter by 11 cm height) containing 50 ml of MS medium (Table 1) at 25°C under cool white fluorescent bulbs (60 μE $m^{-2}s^{-1}$). All treatments were performed in a laminar-flow sterile cabinet at room temperature.

2. Preparation of the transformation vector - *Agrobacterium tumefaciens* containing a p35S GUS INT plasmid (Vancanneyt *et al.* 1990) was utilized. This plasmid carries the NPTII gene coding for kanamycin resistance and the coding sequence of the β-glucuronidase (GUS) *uidA* reporter gene (Jefferson, 1987) interrupted by the IV2 intron (Eches *et al.* 1986). Use of this vector enabled staining for GUS expression immediately after transformation and, at the same time, avoided *Agrobacterial*-derived, GUS-positive background. For transformation experiments, a single colony was picked and resuspended in Suppl. 2YT medium, the ingredients of which are detailed in the following Table 4:

Table 4

Supplemented 2YT liquid medium (Suppl. 2YT medium)

	Amount/liter
Bacto tryptone	16 g
Bacto yeast extract	10 g
NaCL	5 g
Rifampicin	25 mg
Kanamycin	50 mg
Carbenicillin	50 mg
pH brought to 7.0 with NaOH	

The bacteria were cultured for 16 hours on a gyratory shaker (250 rpm) at 28°C. Before co-cultivating the bacteria and the plants, the bacterial culture was diluted with MS medium (Table 1) or booster medium (Table 5, below) to an optical density at 550 nm of 0.6 and brought to pH 4 with HCl. This yielded an *Agrobacterium* preparation suitable for transformation of *Lemnaceae*.

3. Microinjection - *Lemnaceae* plants were transferred to MS medium brought to pH 4, or to a booster medium of the invention having the ingredients as detailed in the following Table 5:

Table 5

Agrobacterium virulence-booster medium of the invention

Component	Amount
Caffeine (Sigma)	150 mg
Fresh cell suspension from *Nicotiana tabacum* (2SH) (prepared as in Aviv and Galun, 1984)	20 ml
MS basal medium (Table 1) pH brought to 4.0 with HCl prior to autoclaving	980 ml

Plants were microinjected under a dissecting microscope using a 1 ml sterile disposable syringe with a G-27 needle, and filled with a preparation of *Agrobacterium* suitable for transformation of *Lemnaceae*. The injections were targeted toward the meristematic zones of the plants in order to bring the bacteria

into close contact with the growing meristem. In each injection, about 20 μl were injected inside the meristematic zone and each zone was injected three times.

4. Co-cultivation - The injected *Lemna* or *Spirodela* plants were co-incubated with the suitably prepared *Agrobacterium* in MS medium (Table 1) brought to pH 4, or in the booster medium of the invention (Table 5), for 48 hours at 25°C under cool white fluorescent bulbs (30 μE $m^{-2}s^{-1}$). Following this, the plants were washed 3 times with sterile distilled water at room temperature and cultured in MS medium (Table 1) supplemented with 400 mg/l claforan.

VI. Standard X-Gluc Staining Procedure

In an 1.8 ml Eppendorf tube, 5 mg of x-gluc (Duchefa Biochemie BV) were dissolved in 150 μl of dimethyl formamide. Then the following staining solution was added: 10 ml of 100 mM Tris 7.0; 15 μl of 500 mM ferrocyanide (stock kept frozen); 15 μl of 500 mM ferricyanide (stock kept frozen) and 100 μl of 10% Triton x-100. The plants were then transferred to a 9 cm Petri dish and 10 ml of staining solition were added. Tubes were incubated overnight at 37°C in darkness. Then the staining solution was discarded and rinsed with distilled water. The GUS positive plants were observed with a binocular microscope.

VII. Callus formation and long-term maintenance of morphogenetic *Spirodela*

Spirodela punctata plants were transferred to SP medium (Table 2) for 5 days at 26°C under continuous fluorescent light (30 μE $m^{-2}s^{-1}$). The plants were placed under a binocular microscope, illuminated from below, and the growing daughter fronds removed, by a plucking motion using a forceps. For callus induction, mother fronds were grown on B-5 medium (Table 6, below) supplemented with 1.0% sucrose, 2 mg/l BA and 50 mg/l of Dicamba. After growing for 3 weeks on this medium, the calli were transferred to B-5 medium supplemented with 2 mg/l 2IP and 10 mg/l 2,4-D. Long-term maintenance of calli was achieved by periodical transfer every 4 weeks to fresh B-5 medium supplemented with 1.0% sucrose, 2 mg/l 2IP and 10 mg/l 2,4-D.

VIII. Rapid regeneration of *Spirodela* plants from calli

Spirodela calli were maintained on B-5 medium (Table 6 below) supplemented with 1.0% sucrose, 3 mg/l 2IP and 10 mg/l 2,4-D. For regeneration, calli were transferred to B-5 medium supplemented with 1.0% sucrose and 2 mg/l 2IP. Fully regenerated *S. punctata* plants were efficiently obtained within 1-2 weeks. *Spirodela* calli and the regenerated plants, were grown at 26°C under continuous fluorescent light (30 μE $m^{-2}s^{-1}$).

Table 6

B-5 medium (modified from Gamborg *et al.*, 1968)

	Amount (mg/l)
KNO_3	2500
$MgSO_4\bullet 7H_2O$	250
$Na_2H_2PO_4\bullet H_2O$	150
$CaCl_2\bullet 2H_2O$	150
$(NH_4)_2SO_4$	134
FeEDTA	28
H_3BO_3	3
$MnSO_4\bullet H_2O$	10
$ZnSO_4\bullet 7H_2O$	2
$Na_2MoO_4\bullet H_2O$	0.25
$CuSO_4\bullet 5H_2O$	0.025
$CoCl_2\bullet 6H_2O$	0.0.25
KI	0.75
Nicotinic acid	1
Thiamine HCl	10
Pyridoxine HCl	1
m-inositol	100
Gelrite	3 g/l
pH brought to 5.8 with NaOH prior to autoclaving	

IX. Semi-automated meristem isolation and transformation

1. Synchronization of *Spirodela* and *Lemna* growth by meristem exposure -

All materials were sterilized and all procedures were carried out under aseptic conditions. Plants (10 g) were harvested in a laminar-flow sterile cabinet by pouring the contents of a culture vessel through a sterilized 10 mesh (1.7 mm pore size) stainless steel sieve. The *Spirodela* or *Lemna* plants were transferred from the top of the sieve with a sterile spoon to a 1 liter-capacity sterile blender, modified to contain 6 razor blades positioned in three different planes (Blumenthal *et al.*, 1993). The blender was filled with 250 ml of distilled, filter-sterilized water and activated for 4 sec. at 17000 rpm. The partially homogenized plants were poured aseptically from the blender through a 20 mesh (800 μ pore size) sterilized Nitex sieve and collected on a 42 mesh (350 μ pore size) sterilized Nitex sieve. Subsequently, a jet of filter-sterilized distilled water (2 atm., 10 liters/min) was directed on top of the 20 mesh Nitex sieve in order to force all of the explant particles smaller than 800 μm to pass through, yielding a particle size population of 350-750 μm. Explant particles larger than 800 μm were transferred back to the blender with a sterile spoon, the blender activated for 3 sec at 17000 rpm, and all sieving and washing steps repeated as above. The combined size population of 350-750 μm, collected on a 42 mesh sterilized Nitex sieve, was subjected to a final sterile water-jet wash. The 350-750 μm sieved explant particles (5 g) were collected by sterile spoon and spaced in a 14 cm diameter sterile petri dish. Throughout this process, all the waste was directed by gravitational force to a 30 liter plastic container situated below the laminar flow cabinet.

2. Co-cultivation followed by recovery period - All materials were sterilized and all procedures were carried out under aseptic conditions. The sieved 350-750 μm explant particles from *Spirodela* or *Lemna*, in the 14 cm diameter petri dish, were resuspended in 15 ml of MS medium (Table 1) brought to pH 4, and then mixed with 15 ml of *Agrobacterium* previously cultured in Suppl. 2YT medium (Table 4) for 24 hrs. at 25°C on a gyratory shaker at 250 rpm. The explant-bacteria mixture was co-cultivated for 1 hr at 25°C and 10 μE $m^{-2}s^{-1}$. Following this, the mixture was transferred by spoon to a 100 mesh (150 μm pore size) sterilized Nitex sieve. The particles excluded by the sieve were washed with 5 ml of MS medium (Table 1) brought to pH 4, and transferred to a 3 liter wide-mouth culture vessel containing 500 ml of the same medium. Co-cultivation continued for 48 hr. at

25°C under continuous fluorescent light (30 μE m^{-2}s^{-1}). At the end of this period, the mixture was sieved through a 100 mesh (150 μ pore size) Nitex sieve, followed by three 200 ml rinses with distilled water. Explant particles with associated bacteria were transferred by spoon to a 3 liter wide-mouth culture vessel containing 500 ml of MS medium (Table 1) supplemented with 400 mg/l claforan and cultured for 5 days at 25°C and 30 μE m^{-2}s^{-1} light. The explants were then sieved as before, followed by separation of the floating material (most of which was living and proliferating) from the sunken, non-vital particles. This was achieved by pouring the explant material together with distilled water, into a 200 ml graduated cylinder and collecting the floating material by a straining spoon. Explants were cultured in 1 liter Erlenmeyer flasks containing 300 ml of SP medium (Table 2) for 3 days.

X. Selection and reporter genes

NPT II Selection and GUS staining were used to determine whether the cells of the treated plants tissue or calli were transformed, i.e. that they contained the transforming DNA. A DNA comprising the caMV 35s promoter was used followed by the *E. coli* NPT II coding sequence, which confers kanamycin resistance, as an expression vector. Stable inheritance of transgenic traits (kanamycin resistance and GUS activity) were assayed on permissive media while the NPT II gene and its products were assayed by PCR and immuno-blotting, respectively.

Example 1:

Transformation of *Lemna* and *Spirodela* plants by microinjection

A color-marker reporter gene (*GUS*) and a gene conferring resistance to the antibiotic kanamycin (*NPT II*) were transferred into the *Lemnaceae* plants (*Lemna gibba* Hurfeish and *Spirodela punctata*) by *Agrobacterium tumefaciens* mediated transformation. This was achieved after suitably preparing the plants and actively promoting DNA transfer into the plant nucleus as specified above.

Booster medium of the invention (Table 5) markedly enhanced *Agrobacterium* virulence against *Lemnaceae*. Applying *Agrobacterium* to *Lemna*

or *Spirodela* plants, maintained for two months in MS medium (Table 1), while omitting the booster medium drastically reduced microinjection-mediated transformation frequencies as shown in the results of the following experiments:

Experiment 1.1:

49 out of 100 microinjected *Lemna* plants maintained in MS medium were GUS positive when the booster medium of the invention was used, while 3 out of 100 were GUS positive when the booster medium was omitted.

Experiment 1.2:

47 out of 100 microinjected *Spirodela* plants maintained in MS medium were GUS positive when the booster medium of the invention was used, while 2 out of 100 were GUS positive when the booster medium was omitted.

Experiments 1.1 and 1.2 thus prove that the booster medium of the invention significantly raises the efficiency of transformation.

Experiment 1.3:

34 out of 100 microinjected *Lemna* plants maintained in MS medium were GUS positive when the booster medium of the invention was brought to pH 4.0; 19 out of 100 were GUS positive when the booster medium was brought to pH 5.2; and 9 out of 100 were GUS positive when the booster medium was brought to pH 7.5.

Experiment 1.4:

31 out of 100 microinjected *Spirodela* plants maintained in MS medium were GUS positive when the booster medium of the invention was brought to pH 4.0; 13 out of 100 were GUS positive when the booster medium was brought to pH 5.2; and 5 out of 100 were GUS positive when the booster medium was brought to pH 7.5.

These results clearly indicate that a pH below about 5.2 raises the efiency of transformation.

The addition of caffeine, a novel agent in transformation protocols, and live tobacco cells (Aviv and Galun, 1984), was found to promote *Agrobacterium* transformation, as shown in the following experiments:

Experiment 1.5:

44 out of 100 microinjected *Lemna* plants maintained in MS medium were GUS positive when co-cultivation was carried out in MS medium brought to pH 4.0, which contained caffeine and live tobacco cells; while only 33 out of 100 were GUS positive when caffeine and tobacco cells were omitted.

Experiment 1.6:

39 out of 100 microinjected *Spirodela* plants maintained in MS medium were GUS positive when co-cultivation was carried out in MS medium brought to pH 4.0, which contained caffeine and live tobacco cells; while only 31 out of 100 were GUS positive when caffeine and tobacco cells were omitted.

Experiments 1.5 and 1.6 thus indicate that addition of caffeine and live tobacco cells to the booster medium of the invention raises the transformation efficiency.

Example 2: Development of plants from explant particles

The semi-automated *Lemnaceae* blending process resulted in a purified fraction of explant particles 350-750 μm in size, from either *Lemna* or *Spirodela*, which represented approximately 50% of the total starting material. Among the 350-750 μm particles were explant particles which were seen to contain undamaged meristematic zones, from which new plants vigorously grew. Explant particles smaller than 150-350 μ gave drastically reduced number of actively growing plants. The meristem-containing explants remained green, floated and grew to maturity. All other explant particles rapidly turned yellowish-brown, eventually bleaching entirely, and most sunk to the bottom of the culture vessel. The massive death of the non-meristematic explants sections did not inhibit the normal development of new plants, which developed from the meristem-containing explant sections into mature *Lemnaceae* plants morphologically indistinguishable from non-blended

plants, as demonstrated in the following experiments:

Experiment 2.1:

Forty-eight hours after the blending process, 600-800 explant particles out of a total of 80,000 gave rise to tiny green *Spirodela* plants. The incipient colonies each contained 1-2 plants not longer than 1 mm. Six days after blending, these colonies consisted of 3 plants, each 2-3 mm long, and 2-3 newly developed roots (5-6 mm in length). Nine days after blending, the plants reached an average size of about 4-5 mm long and a shape both comparable to that of non-blended control *Spirodela*. At this stage, colonies contained 5-7 fronds and 5-6 fully elongated roots. These plants were further subcultured for at least 5 generations. The average biomass doubling time was 2 days and was not distinguishable from that of control plants. No somaclonal variation was observed.

Experiment 2.2:

Forty-eight hours after the blending process, 300-500 explants particles out of a total of 80,000 gave rise to tiny green *Lemna* plants. The incipient colonies each contained 1 plant not longer than 1 mm. Six days after blending, these colonies consisted of 2 plants, each 2-3 mm long, and 2 newly developed roots (1 mm in length). Nine days after blending, these new plants reached the average size of about 7 mm long and had a shape comparable to that of non-blended control *Lemna*. At this stage, colonies contained 4 plants and 2-4 roots. These plants were further subcultured for at least 5 generations. The average biomass doubling time was 2 days and was not distinguishable from that of control plants. No somaclonal variation was observed.

Example 3:

Analysis of *Lemna* and *Spirodela* plants transformed by the semi-automated meristem Lemnaceae blending process and by the transformation procedure

A. GUS positive staining in transformed *Lemnaceae* plants following semi-automated meristem exposure and transformation.

Following transformation with *Agrobacterium* harboring the β-glucuronidase (GUS) *uidA* reporter gene, explants were cultured for 3-7 days and then stained for GUS activity. The following are results of two such experiments:

Experiment 3.1:

Sixteen hours after immersing *Agrobacterium*-transformed *Spirodela* colonies in a GUS reaction mixture (Jefferson, 1987), 120 of 600 colonies in one repetition, and 400 of 800 colonies in another, exhibited blue sectors, indicating that 20-50% of the explants were transformed. The size of transformed sectors ranged from 0.01 to 1 mm^2. Untransformed control plants did not exhibit any GUS staining. In 2 out of the 600 and 16 of the 800 colonies (0.3-2%), daughter generation plants were stained systematically blue. This indicated that the meristematic zones from which the daughter plants regenerated, had been transformed. In some colonies, the mother generation plant remained unstained while systemic GUS staining was observed in some of the daughter generation plants. This indicates that mass meristem exposure of *Spirodela* plants can lead to meristem-targeted transformation.

Experiment 3.2:

Sixteen hours after immersing *Agrobacterium*-transformed *Lemna* colonies in a GUS reaction mixture (Jefferson, 1987), 60 of 300 colonies in one repetition, and 250 of 500 colonies in another, exhibited blue sectors, indicating that 20-50% of the explants were transformed. The size of transformed sectors ranged from 0.01 to 1 mm^2. Untransformed control plants did not exhibit any GUS staining. In 1 out of the 300 and 5 of the 500 colonies (0.3-1%), daughter generation plants were stained systemically blue.

B. Integration of the kanamycin resistance gene

In order to verify insertion and integration of the transferring DNA from *Agrobacterium*, total DNA was extracted from *Lemna* and *Spirodela* plants that were previously selected for positive GUS staining. The DNA was amplified in a PCR reaction (annealing at 55°C) with the following primers of the NPT II coding regions:

1. 5′ GCACGAGGTTCTCCGGCCGCTTGGG 3′;
2. 5′ GAAGGCGATGCGCTGCGAATCGGG 3′.

These primers produce a 780 bp fragment within the NPT II gene. The PCR reaction product were electrophoresed on an agarose gel (0.8%) and stained with ethidium bromide. GUS-positive *Lemna* and *Spirodela* plants exhibited the expected band at the expected size for the NPTII transgene. Untransformed controls of *Lemna* or *Spirodela* plants did not contain this band. The same band at the same migration position was evident also in DNA isolated from the Ti plasmid of *Agrobacterium*, which was electrophoresised on the same gel as a positive control. These results verified that *Agrobacterium* is capable of genetically transforming *Lemna* and *Spirodela* plants.

C. Transformation of specific organs in an intact *Lemnaceae* plant

In 5% of the transformed population, expression of the introduced GUS gene was detected only in the root system. In these cases, GUS expression was detected all over the root system. This is of importance in cases where it is of interest to express the introduced gene in only a defined part of the plant such as root tissue.

Example 4 Identification of *Agrobacterium* strains which have a specificity towards transformation of meristemic tissue in *Lemnaceae*

Spirodela punctata and *Lemna gibba* var. Hurfeish plants were maintained in SP medium (Table 2) under standard growth conditions (Procedure II). Using the standard transformation procedure (Procedure III), intact plants were co-cultivated with 5 different *A. tumefaciens* strains (EHA105 [Xiu-Qing Li *et al.*, 1992]; EHA101 [Hood *et al.*, 1987]; GVE3103 [Deblaere *et al.*, 1985]; LBA4404

[Ooms *et al.*, 1982]; and C58 [Van Larebeke *et al.*, 1974]) each harboring Ti plasmid pME504 (shown in Fig. 1). This plasmid carries: the *npt*II gene, conferring resistance to the antibiotic kanamycin, under the control of the nopaline synthase promoter; the *bar* gene, conferring resistance to the herbicide BASTA (Thompson *et al.*, 1987), under the control of the 35S-CaMV promoter; and the *uid*A gene interrupted by an intron (Vancanneyt *et al.*, 1990), coding for the GUS reporter, also under the control of the 35S-CaMV promoter. GUS expression (Procedure VI) was determined by scoring blue spots. The tissue specificity of the different *A. tumefaciens* strains was determined by scoring the distribution of blue spots in the *Lemnaceae* plants. The data are summarized in Table 7, below.

The method involved wounding *Spirodela punctata* and *Lemna gibba* Hurfeish plants, co-cultivating the plants with 5x10^8 bacteria ml^{-1}, vacuum infiltration (30 mbar, 5-10 min) and further co-cultivation for 4 hr. Fronds were assayed for GUS expression 10 days after co-cultivation.

The results indicate that GUS expression in *S. punctata* co-cultivated with *A. tumefaciens* strains EHA105, EHA101 and GVE3103 was restricted mainly to daughter fronds arising from meristematic tissue, while GUS expression in *S. punctata* co-cultivated with *A. tumefaciens* strains LBA4404 and C58 was restricted mainly to wounded areas of the mother frond.

Table 7

A. tumefac.	Ti plasmid	**GUS expression (% of fronds)** **Spirodela**		**Lemna**	
strain	type	mother frond	daughter frond	mother frond	daughter frond
EHA105	agropine	6	23	1	7
EHA101	agropine	7	22	1	5
CVE3101	octopine	3	18	0	0
LBA4404	octopine	10	3	0	0
C58	none	8	1	0	0

Example 5: **Identification of *Agrobacterium* strains which specifically target and transform wounded tissue in *Lemnaceae***

Spirodela punctata and *Lemna gibba* Hurfeish were maintained in SP medium (Table 2) under standard growth conditions (Procedure II). Using standard transformation procedure (Procedure III), intact plants were co-cultivated with 5 different *A. tumefaciens* strains each harboring the Ti plasmid pME504 (shown in Fig. 1). GUS expression was determined by scoring blue spots. The tissue specificity of the different *A. tumefaciens* strains was determined by the distribution of spots in the *Lemnaceae* plants. GUS expression in *S. punctata* co-cultivated with *A. tumefaciens* strains LBA4404 and C58 was restricted mainly to wounded areas of the mother frond, while GUS expression in *S. punctata* co-cultivated with *A. tumefaciens* strains EHA105, EHA101 and GVE3103 was restricted mainly to daughter fronds arising from meristematic tissue as shown in Table 7, above.

Example 6 Use of vacuum infiltration for increasing efficiency of transformation of *Lemnaceae* by *Agrobacterium*

Spirodela punctata and *Lemna gibba* Hurfeish plants were maintained in SP medium (Table 2) under standard growth conditions (Procedure II). Intact plants were co-cultivated with *A. tumefaciens* strain EHA105 harboring Ti plasmid pME504 using the standard transformation procedure (Procedure III) with and without vacum infiltration (30 mbar, 5-10 min). Transformation efficiency was determined by scoring GUS expression (blue spots). The data are shown in Table 8 below.

The method involved wounding *Spirodela punctata* var. Helgm and *Lemna gibba* var. Hurfeish plants, co-cultivating them with 5×10^8 bacteria ml^{-1} (*A. tumefaciens* strain EHA105 harboring Ti plasmid pME504) and vacuum infiltration (30 mbar for 5-10 min). Control plants were wounded and co-cultured as above but without vacuum infiltration. Plants were assayed for GUS expression 10 days after co-cultivation.

The data shows an increase in transformation efficiency of 61% for *Spirodela* and 400% for *Lemna* following vacuum infiltration.

Table 8

GUS expression (% fronds)			
	Control	Vacuum infiltrated	% increase
Spirodela	18	29	61
Lemna	2	8	400

Example 7: Method for increasing efficiency of *Lemnaceae* transformation by *Agrobacterium* by exposing the meristematic zones of the mother frond

In order to partially expose the meristematic zones of *Lemnaceae* mother fronds to *Agrobacteria*, plants were placed under a binocular microscope, illuminated from below, and the growing daughter fronds removed; for example, by a plucking motion using a forceps. This procedure had no effect on the viability of the treated fronds. An experiment involving 500 *Spirodela punctata* plants, half of which had their meristematic zones exposed by daughter frond removal, resulted in an increase of GUS expression in meristematic zones from 14% (not treated) to 23% (meristematic zone exposed).

Example 8: Method for increasing efficiency of *Agrobacterium* transformation of *Lemnaceae* by direct dissection and exposure of mother frond meristematic zones

Following removal of the daughter fronds from the meristematic pockets of the mother frond, the mother frond was longitudinally dissected under the binocular microscope in order to fully expose its meristematic zones. An experiment was performed in which the daughter fronds were removed from 500 *Spirodela punctata* plants. Following this, 250 of these plants were also longitudinly dissected. GUS expression was monitored 10 days following co-cultivation with *A. tumefaciens* EHA105 harboring Ti plasmid pME504. GUS expression was observed in 33% of the longitudinally dissected plants, compared with 25% in the non-dissected ones.

Example 9: Method for increasing stability of *Agrobacterium* transformation of *Lemnaceae* by direct dissection and exposure of mother frond meristematic zones

The method involved treating five hundred *Spirodela punctata* as described in Example 8. For all plants, daughter fronds were first removed. In addition, one half of the mother fronds were, longitudinally dissected and co-cultivated with 5×10^8 bacteria ml^{-1} *A. tumefaciens* strain EHA105 harboring Ti plasmid pME504. Plants were vacuum infiltrated (30 mbar for 5-10 min). Plants were assayed for GUS expression 5, 10 and 15 days after co-cultivation and their filial relationship to the dissected mother frond was recorded. The results are shown in Table 9.

Table 9

GUS expression (% fronds)						
Treatment	Mother frond	Daughter front generation				
	F_0	F_1	F_2	F_3	F_4	F_5
Longitudinally dissected	0	19	5	2	1	0.2
Non dissected	6	15	3	1	0	0.0

An increase in the stability of GUS expression meristematically transformed from one filial generation to the next was obtained as evident from Table 9.

Example 10: Utilization of *Lemnaceae* extracts for increasing efficiency of transformation

Spirodela punctata plants were maintained in SP medium (Table 2) under standard growth conditions (Procedure II). Using standard transformation procedure (Procedure III), plants were co-cultivated with *A. tumefaciens* strain EHA105 harboring Ti plasmid pME504. Plants were wounded or left intact (non-wounded). Thereafter, they were co-cultivated with *A. tumefaciens* in SP medium and supplemented for various periods of time with an extract from *Spirodela* plants. Transformation efficiency was determined by scoring GUS expression (blue spots).

The method involved maintaining 500 *Spirodela punctata* plants treated as described in Procedure III. Of these 500 plants, 250 were wounded. All plants were co-cultivated with $5x10^8$ bacteria ml^{-1} (*A. tumefaciens* strain EHA105 harboring Ti plasmid pME504), and exposed to an extract from *Spirodela*. The extract was prepared by homogenizing 20 g of *Spirodela* plants from a one-week old culture in 50 ml phosphate buffer (pH 7.0). The homogenate was centrifuged (10 min. 10000 rpm) and the supernatant filter sterilized. The resulting *Spirodela* extract was applied for 4 hr during co-cultivation, or for this period plus the subsequent 10 days. Control plants were not exposed to the extract. All plants were assayed for GUS expression 10 days after co-cultivation.

The data summarized in Table 10 show that the presence of the *Spirodela* extract enhanced the transformation efficiency of the non-wounded plants.

Table 10

Incubation with *Spirodela* extract (h)	**GUS expression (% of fronds)**	
	Wounded	**Non-wounded**
0	16	8
4	17	10
240	16	14

Example 11 Demonstration of transformability of several species from a number of genera of *Lemnaceae*

All plants were maintained in SP medium (Table 2) under standard growth conditions (Procedure II). Using standard transformation procedure (Procedure III), plants were co-cultivated with *A. tumefaciens* strains EHA105 harboring Ti plasmid pME504. Table 11 summarizes the percentage of plants from different *Lemnaceae* species expressing GUS 10 days after co-cultivation. Gus expression in F_1 daughter fronds was determined for three different genera of *Lemnaceae*: (A) *Spirodela (B)*; (b) *Lemna*; and (C) *Wolffia*, all inoculated with *A. tumefaciens* strain EHA105 (pME504).

The method involved wounding plants, co-cultivating them with $5x10^8$ bacteria ml^{-1} (*A. tumefaciens* strain EHA105 harboring Ti plasmid pME504),

vacuum infiltration (30 mbar for 5-10 min.) and then further co-cultivation for 30 min. Plants were assayed for GUS expression (blue stain) 10 days after co-cultivation.

The results demonstrate the general applicability of the method of the invention for *Lemnaceae* transformation.

Table 11

Genus	Species	Strain	% stained plants
Spirodela	*intermedia*	7797	3
Spirodela	*punctata*	8717	92
Spirodela	*punctata*	Hegelm	27
Lemna	*obscura*	7325	12
Lemna	*obscura*	7780	14
Lemna	*gibba*	Hurfeish	8
Lemna	*gibba*	G-3	5
Wolffia	*brasiliensis*	8743	0.1
Wolffia	*australiana*	8730	9

Example 12: Transformed *Lemnaceae* expressing antibiotic resistance

Spirodela punctata plants obtained by standard transformation procedure (Procedure III) were placed in SP medium (Table 2) containing 2 μg/ml kanamycin. In non-transformed control cultures, newly emerging plants grew white in the presence of the antibiotic. However, following transformation and culturing in the presence of kanamycin (2 μg/ml for two months), three out of 500 (Experiment 1), and three out of 300 (Experiment 2) newly emerging plants were green and resistant to the bleaching effects of the antibiotic. This indicated that the *NPTII gene* was present and expressed in the green, resistant plants. The following plants were monitored: a kanamycin resistant clone 10 generations after the start of Experiment 2; a kanamycin sensitive colony (Kn-) with bleached daughter fronds, which did not develop further in the presence of kanamycin during the two months of the experiment; a non-transformed control showing a colony with bleached daughter fronds after 7 days exposure to 2 μg/ml kanamycin. Staining of a sample taken from the kanamycin resistant clone two months after the start of Experiment

2 showed blue GUS staining in more than 80% of the fronds. The results are shown in Table 12.

The method involved wounding plants, co-cultivating them with $5x10^8$ bacteria ml^{-1} (*A. tumefaciens* strain EHA105 harboring Ti plasmid pME504) for 30 min. and vacuum infiltration (30 mbar, for 5-10 min). Transformed *Spirodela punctata* plants were grown in SP medium (Table 2) supplemented with 2μg/ml kanamycin for 2 to 5 weeks. Six green plants resistant to kanamycin, and a sampling of bleached ones, were assayed for GUS expression.

The data summarized in Table 12 indicate that the green, antibiotic-resistant plants were indeed transformed.

Table 12

Supplement to SP medium	**GUS expression (% fronds)**	
	Green plants	Bleached plants
Kanamycin (2 μg/ml)	83	0
BASTA (2 μg/ml)	76	0

Example 13: Transformed *Lemnaceae* carrying herbicide resistance

Five hundred plants of *Spirodela punctata 8717* were co-cultivated with *A. tumefaciens* EHA105 (pME504), carrying: the *npt*II gene conferring resistance to the antibiotic kanamycin; the *bar* gene conferring resistance to the herbicide BASTA; and the *uid* A gene (interrupted by an intron (Vancanneyt *et al.*, 1990) coding for the GUS reporter. Co-cultivated plants were grown in SP medium (Table 2) supplemented with 2 μg/ml BASTA for 5 weeks. The plants were periodically transferred to fresh BASTA supplemented medium every 2 weeks. Under these conditions, control plants failed to grow and eventually bleached completely after 16 days. Thirty four out of 500 plants co-cultivated with *A. tumefaciens* EHA105 (pME504) were resistant to the bleaching effects of the herbicide. This indicated that the *bar* gene was present and expressed in these green, growing plants. The following plants were monitored: green plants from a herbicide resistant clone; a BASTA sensitive, bleached plant (BASTA-) which

failed to develop F_1 daughter fronds; and a control (non-transformed controled showing a bleached plant. The plants were monitored 16 days after co-cultivation. Staining of a sample taken from the BASTA resistant clone 25 days after the start of the experiment showed blue GUS staining in more than 75% of the fronds. The data is summarized in Table 12. The results indicate that the green, herbicide-resistant plants, repeatedly selected on fresh BASTA supplemented medium, were indeed transformed.

Example 14: Transformed *Lemnaceae* carrying fluorescence reporter genes

Spirodela punctata plants were maintained in SP medium (Table 2) under standard growth conditions (Procedure II). Using the standard transformation procedure (Procedure III), plants were co-cultivated with *A. tumefaciens* EHA105 (pME506) carrying the *npt*II gene conferring resistance to the antibiotic kanamycin; the *bar* gene conferring resistance to the herbicide BASTA; and the *luc* gene coding for the firefly luciferase reporter LUC. In parallel, other plants were co-cultivated with *A. tumefaciens* EHA105 (pME508) carrying the *npt*II gene conferring resistance to the antibiotic kanamycin; the *bar* gene conferring resistance to the herbicide BASTA; and a gene coding for the green fluorescence protein (GFP) of *Aequorea victoria*. Expression of the fluorescence reporter genes in the *Spirodela* plants was determined (Millar *et al.*, 1992; Chiu *et al.*, 1996) 10 days after co-cultivation. Expression of GFP was found throughout the frond, when viewed at a magnification of 200 times.

Example 15: Expression of multiple foreign genes in one transformed *Lemnaceae* plant

S. punctata plants were co-cultivated with *A. tumefaciens* EHA105 (pME504), carrying: the *npt*II gene conferring resistance to the antibiotic kanamycin; the *bar* gene conferring resistance to the herbicide BASTA; and the *uid*A gene (interrupted by an intron (Vancanneyt *et al.*, 1990) coding for the GUS reporter. Transformed plants were grown in SP medium (Table 2) in the presence of either 2 μg/ml kanamycin or 2 μg/ml BASTA for 2-5 weeks. Resistant green

plants, as well as a sample of bleached plants, were assayed for GUS expression. The results, summarized in Table 12, demonstrate a high correlation between antibiotic- or herbicide-resistant green plants, and GUS-expressing plants. This demonstrates co-expression of multiple genes in transformed *Lemnaceae*.

Example 16: Identification of a high-efficiency-transformation strain of *Lemnaceae*

Experiments demonstrated a high frequency of GUS staining of a representative population of transformed *Spirodela punctata 8717* four days after transformation. The transformation rate for this strain is > 90% using the standard transformation procedure (Procedure III).

Example 17: Stable, non-chimeric transformation of *Lemnaceae*

Spirodela punctata 8717 plants were maintained in SP medium (Table 2) under standard growth conditions (Procedure II). Using the standard standard transformation procedure (Procedure III), plants were co-cultivated with *A. tumefaciens* EHA105 (pME504), carrying the *npt*II gene conferring resistance to the antibiotic kanamycin; the *bar* gene conferring resistance to the herbicide BASTA; and the *uid*A gene interrupted by an intron (Vancanneyt *et al.*, 1990) coding for the GUS reporter (Fig. 1). The expression of the GUS reporter gene in transformed plants was periodically determined by sampling the population at 4, 10 and 35 days after co-cultivation. Representative examples show more than 7 successive generations (attached by their stipes) of transformed plants expressing GUS throughout their tissues. This indicates stable, non-chimeric transformation of entire *Lemnaceae* plants over several generations and an extended period of time.

Example 18: GUS expression in *Lemnaceae* plants transformed with a promoterless *uid*A gene, indicating integration of foreign DNA into the *Lemnaceae* chromosome

S. punctata plants were co-cultivated with *A. tumefaciens* GV3103 (pVCGUS) (Koncz *et al.*, 1989) which contains a promoterless GUS construct (the *uid*A gene interrupted by an intron). Transformed plants, expressing GUS seven days after

co-cultivation, were monitored. Promoterless GUS expression is only possible if the *uid*A gene was integrated into the *Lemnaceae* chromosome adjacent to endogenous *Lemnaceae* regulatory sequences. As a result of random integration of the *uid*A gene, variability in the level of GUS expression would be expected among different transformation events. The results of the experiment show 3 different intensities of GUS blue stain, the most intense of which matches the typical strong intensity found in transformed plants under the 35S-CaMV promoter.

Table 13 summarizes the results for over 12,000 plants in 4 different experiments (each with 3 different strains of *A. tumefaciens*) which were scored for GUS staining 10 days after co-cultivation. Four thousand plants were co-cultivated with *A. tumefaciens* GV3103 lacking a binary vector (Control). None stained blue, indicating no endogenous GUS activity. Four thousand plants were co-cultivated with *A. tumefaciens* GV3103 harboring a promoterless construct (pVCGUS). Up to 1.4% of the plants stained blue, indicating a highly significant level (versus Control) of integration of the promoterless *uid*A gene into the *Lemnaceae* chromosome adjacent to endogenous *Lemnaceae* regulatory sequences. Four thousand plants were co-cultivated with *A. tumefaciens* GV3103 (pME504), harboring the *uid*A gene under the control of the 35S-CaMV promoter. Twenty eight percent of plants stained blue, indicating a relatively high level of expression using this heterologus promoter.

The method involved co-cultivation of approximately 3000 *Spirodela punctata* plants (6 gr fresh weight) in each transformation experiment (1000 plants for each of 3 constructs). Plants were scored for GUS expression 10 days after co-cultivation. The results were categorized according to the intensity of blue color: light (+), medium (++) and dark (+++).

The data in Table 13 show that the variability in intensity of GUS staining among plants co-cultivated with *A. tumefaciens* GV3103 (pVCGUS) was considerably higher than with those co-cultivated with *A. tumefaciens* GV3103 (pME504). The relatively low percentage and variability (versus Control) in intensity of GUS staining plants co-cultivated with the promoterless GUS construct are explained by random integration of this gene into the *S. punctata* chromosome and expression by various endogenous *Spirodela* promoters.

Table 13

GUS expression (No. of fronds)												
	pVC-GUS				pME504				No. Ti plasmid			
	+	++	+++	total	+	++	+++	total	+	++	+++	total
I	7	3	2	12	0	14	231	245	0	0	0	0
II	1	1	3	5	0	10	271	281	0	0	0	0
III	9	5	0	14	0	6	201	206	0	0	0	0
IV	3	1	2	6	0	21	209	230	0	0	0	0
Sum				37				962				0
% (ave.)	0.9				24				0			

Example 19: Development of an *Agrobacterium*-mediated, *in-planta*, non-chimeric transformation of *Lemnaceae* without *de novo* regeneration

It was demonstrated that it is possible to develop a novel transformation system in *Lemnaceae* which does not require tissue culture or *in vitro* regeneration procedures. The procedures enable *in planta*, direct meristem targeting of the transforming vehicle. In this novel system, a meristem is transformed and has the capability to continue growing and thus form the next generation. Moreover, no selection pressure is needed to avoid the growth of non-meristematic tissue within the same *Lemnaceae* plant. Examples of the validity of this approach can be seen in the results of Examples 1, 4, 6 and 10 through 18.

Example 20 Method for long-term maintenance of morphogenetic *Spirodela* calli

Transient callus formation and short-term calus maintence was previously reported in 2 species of *Lemna* (Chang and Chiu, 1978a, 1978b). Using one of these authors' callusing media (Murashige and Skoog, 1962), supplemented with 3% sucrose, 1 mg/l 2IP and 10 mg/l 2,4-D), calli were obtained from *Spirodela punctata* but callus development was arrested within 14 to 21 days. During this period, dramatic accumulation of starch (measured as an increase in iodine staining material) within the calli were observed. These calli bleached and

eventually died, apparently due to starch poisoning. Long term maintenance is a prerequisite to transformation at the callus level since screening and/or selection steps are needed.

Conditions for long term maintenance of *Spirodela* calli were sought. Several different media (MS medium (Table 1), B-5 (Table 5), and concentrations of sucrose (3.0, 1.0, 0.5%) were studied in combination with a number of phytohormones (2IP; 2,4-D; Dicamba; BA, Zeatin). B-5 medium supplemented with a low concentration (1.0%) of sucrose was found to best promote long term maintenance of *Spirodela* calli under several hormonal combinations. The data in Tables 14, 15 and 16 summarize percentage callus formation and long term maintenance under several hormonal combinations. The ultimate method for callus formation and long term maintenance of morphogenetic *Spirodela* calli is given in Procedure VII. It uniquely demands a low concentration of sucrose combined with particular hormonal combinations. Using this procedure, green, growing calli were maintained for more than three months.

The method involved culturing separated *Spirodela punctata* var. Helgm fronds on B-5 medium supplemented with 1% sucrose and various concentrations of 2IP and 2,4-D as indicated. Callus formation was monitored after 8 weeks using a dissecting microscope. Calli were subcultured to fresh medium every 4 weeks. The results are shown in Table 14:

Table 14

2 IP (mg/l)	2,4-D (mg/l)	Callus formation[a] No. (%)	Long term maintenance[b] No. (%)
2	2	5 (10)	0 (0)
2	10	134 (90)	48 (34)
2	50	3 (15)	0 (0)
10	2	2 (6)	0 (0)
10	10	4 (10)	0 (0)
10	50	0 (0)	0 (0)

[a] Data scored after 8 weeks of culture
[b] Data scored after 12 weeks of culture

In another experiment separated *Spirodela punctata* fronds were cultured on B-5 medium supplemented with 1% sucrose and various concentrations of BA and Dicamba as indicated. Callus formation was monitored after 3 weeks using a dissecting microscope. Calli were subcultured to fresh medium every 3 weeks. The results are shown in Table 15:

Table 15

BA (mg/l)	Dicamba (mg/l)	Callus formation[a] No. (%)	Long term maintenance[b] No. (%)
2	2	0 (0)	0 (0)
2	10	13 (23)	0 (0)
2	50	175 (100)	151 (86)
10	2	0 (0)	0 (0)
10	10	0 (0)	0 (0)
10	50	0 (0)	0 (0)

[a] Data scored after 3 weeks of culture
[b] Data scored after 6 weeks of culture

In another experiment separated *Spirodela punctata* fronds were cultured on B-5 medium supplemented with 1% sucrose and various concentrations of Zeatin and Dicamba as indicated. Callus formation was monitored after 3 weeks using a dissecting microscope. Calli were subcultured to fresh medium every 3 weeks. The results are shown in Table 16:

Table 16

Zeatin (mg/l)	Dicamba (mg/l)	Callus formation[a] No. (%)	Long term maintenance[b] No. (%)
2	2	0 (0)	0 (0)
2	10	0 (0)	0 (0)
2	50	10 (80)	7 (70)
10	2	0 (0)	0 (0)
10	10	0 (0)	0 (0)
10	50	13 (76)	6 (46)

[a] Data scored after 3 weeks of culture
[b] Data scored after 6 weeks of culture

Example 21: Method for producing highly-regenerative *Spirodela* calli

The unique method for production of *Spirodela* calli is described in Procedure VII. By using this procedure in conjunction with Procedure VIII, regenerated *S. punctata* plants were efficiently obtained (Table 17 below). The combination of the two procedures represents the method for producing highly-regenerative *Spirodela* calli.

Calli of *Spirodela punctata* var. Helgm, maintained for 7 to 16 weeks on B-5 medium supplemented with 1% sucrose, 2 mg/l 2IP and 10 mg/l 2,4-D, were transferred to B-5 medium supplemented with 1% sucrose and different concentrations of 2IP. Regenerated plants were visually scored after 2 weeks.

Table 17

2 IP	**Calli**	**Regenerating calli**	
(mg/l)	No.	No.	(%)
0	30	26	87
2	30	22	73
10	30	0	0

Example 22: Method for rapid and highly-efficient regeneration of *Lemnaceae* plants from calli

Regeneration of frond-like structures from calli of *Lemna perpusilla* has been reported; however, the authors state that they did not observe further development of these frond-like structures even after 2 months (Chang and Hsing, 1978). In a further study, regeneration of plants from calli of *Lemna gibba* (Chang and Chiu, 1978) was reported. However, the procedure required 2 months to obtain an asexual propagating plant Chang and Chiu, 1978). Regeneration of *Spirodela* plants from calli has never been reported. Using the novel methodology of the invention for plant regeneration, intact, regenerated *S. punctata* plants were efficiently obtained within 1-2 weeks. The uniquely rapid method for *Lemnaceae* plant regeneration is given in Procedure VIII.

Example 23: Method for rapid and highly-efficient regeneration of true-to-type *Spirodela* plants from calli

Using the methodology for plant regeneration (Procedure VIII), > 90% of the regenerated *S. punctata* plants visually appeared true-to-type after 3 weeks of growth in SP medium. *S. punctata* plants, viewed after 3 months of growth under standard conditions, continued to appear true-to-type. When compared with their parental progenitor with respect to growth rate, size and frond morphology no significant differences were found.

Example 24: Method for increasing genetic diversity through calli in regenerating *Spirodela* plants

The ability to increase the genetic diversity in *Spirodela* is important since in several species, propagation is strictly vegetative (Landolt and Kandeler, 1987). Using the methodology for plant regeneration (Procedure VIII), several regenerated plants visually appeared aberrant and after 3 weeks of growth under standard condtitions, continued to appear aberrant. When compared with their parental progenitor, significant differences were found in size (smaller), growth rate (slower) and morphology (frond shape).

Example 25: Transformation of *Lemnaceae* calli by *Agrobacterium*

Spirodela calli were maintained on B-5 medium (Table 6) supplemented with 1.0% sucrose, 2 mg/l 2IP and 10 mg/l 2,4-D. Five hundred calli were co-cultivated with *Agrobacterium* harboring the pME504 plasmid. Following 2 days of co-cultivation, the calli were transferred to fresh medium supplemented with 30 mg/l kanamycin and 300 mg/ml carbenicillin. After 15 days, 488 calli were fully bleached. The remaining 12 green calli were transferred to fresh medium supplemented with 30 mg/l kanamycin. Three calli remained green following two additional subcultures (2 months) on fresh media containing kanamycin as above. Green calli which were maintained for more than two months on B-5 medium supplemented with 1.0% sucrose, 2 mg/l 2IP, 10 mg/l 2,4-D and 30 mg/l kanamycin were monitored. The results indicate that the persistent, green calli

were resistant to kanamycin as a result of the expression of the introduced genes.

Example 26: Production of transgenic calli from *Agrobacterium*-infected intact *Lemnaceae* plants

Spirodela punctata plants were maintained in SP medium under standard growth conditions (Table 2). Using the standard transformation procedure (Procedure III), plants were co-cultivated with *A. tumefaciens* strain EHA105 harboring Ti plasmid pME504. Two days following the transformation the intact plants were cultured on B-5 medium supplemented with 10 mg/l Dicamba, 2 mg/l BA and 30 mg/l kanamycin (Procedure VII). Two green, compact calli resistant to kanamycin develoiped from meristematic regions after 25 days. The green growing calli were dissected from the original tissue and further subcultured on fresh medium containing 30 mg/l kanamycin for an additional 20 days. Following this transfer as well, the 2 calli remained green. The results indicate that the persistent green calli were resistant to kanamycin as a result of the introduced genes.

Example 27: Regeneration of transgenic *Lemnaceae* plants from *Agrobacterium*-transformed calli

Spirodela punctata 8717 plants were maintained in SP medium under standard growth conditions (Table 2). Usng the standard transformation procedure (Procedure III), plants were co-cultivated with *A. tumefaciens* strain EHA105 harboring Ti plasmid pME504. Two days following the transformation, the intact plants were cultured on B-5 medium supplemented with 10 mg/l Dicamba, 2 mg/l BA and 30 mg/l kanamycin (Procedure VII). Ten calli, resistant to kanamycin, developed from meristematic regions after 25 days. The green calli were transferred to B-5 medium supplemented with 1% sucrose, 2 mg/l 2IP and 30 mg/l kanomycin. A green regenerant plant was scored after 2 weeks. This plant was transferred to the same media approximately every 2 weeks for a period of 8 months, giving rise to numerous kanamycin-resistant vegetative offsprings, hereafter designated as clone ME11. Clone MEll has been propagated as green and kanamycin resistant in the above media for more than 65 generations.

The ability to obtain kanamycin resistant calli has been demonstrated either by production of transgenic calli from *Agrobacterium*-infected *Lemnaceae* plants (Example 25), or by production of transgenic calli from *Agrobacterium*-infected *Lemnaceae* calli (Exampleas 26). Since kanamycin resistant calli can be produced and true-to-type *Lemnaceae* plants can be efficiently and readily regenerated from calli (Procedure VIII), the technology for producing transformed *Lemnaceae* plants originating from antibiotic resistant calli has been demonstrated.

Example 28: Verification of the long-term expression and stability of the introduced trait

Spirodela punctata 8717 plants were maintained in SP medium under standard growth conditions (Table 2). Clone ME11, transformed as in Example 27, was propagated as green and kanamycin resistant in SP medium supplemented with 2mg/l kanamycin for approximately 40 generations. In order to verify long-term expression and stability of the introduced traits, clone MEll was subcultured on the same medium lacking kanamycin for approximately 60 generations. Clone ME11 was then evaluated for either kanamycin or BASTA resistance by subculturing in SP medium supplemented with either 2mg/l kanamycin or 1.5mg/l BASTA with or without 1% sucrose for 5-10 genrations.

Control, non-transformed Spirodela punctata 8717 plants bleached and eventually died. Clone ME11 plants remained green and retained their normal growth.

These results demonstrate stable expression of kanamycin resistance in ME11 plants in spite of removal from selection pressure for more than 60 genrations. They also demonstrate stable expression of BASTA resistance in spite of a lack of any selection pressure for this trait.

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CLAIMS:

1. A genetically stable, transformed *Lemnaceae* plant and progency thereof.

2. A transformed *Lemnaceae* plant according to Claim 1, of the genera *Spirodela, Lemna* and *Wolffia*.

3. A transformed *Lemnaceae* plant according to Claim 2, being *Spirodela punctata* of strain 8717.

4. An antibiotic resistant transformed *Lemnaceae* plant according to Claims 1 to 3.

5. A transformed *Lemnaceae* plant according to Claim 4, being resistant to kanamycin.

6. A herbicide resistant transformed *Lemnaceae* plant according to Claims 1 to 3.

7. A transformed *Lemnaceae* plant according to Claim 4, being tolerant to oxynil herbicides, to glyphosate and EPSPS inhibitor herbicides, to glufosinate or to HPPD inhibitors.

8. A transformed *Lemnaceae* plant according to Claims 1 to 7, capable of expressing two or more foreign genes.

9. Use of the plant according to Claim 1, for the production of chemical or biological products.

10. Use according to Claim 9, for the production of polypeptides, proteins, carbohydrates, lipids, alkaloids, pigments or vitamins.

11. A chemical or biological product obtained by the use according to Claim 9 or 10.

12. A method for the stable genetic transformation of *Lemnaceae* plants which comprises: incubating *Lemnaceae* plants and/or tissue with *Agrobacterium* cells containing a transforming DNA molecule, whereby cells in said plant tissue become stably transformed with said DNA.

13. A method according to Claim 12, wherein the *Agrobacterium* cells are capable of specifically targeting the plant's meristematic tissue.

14. A method according to Claim 13, wherein the *Agrobacterium* cells are

A. tumefaciens strains EHA105, EHA101 and GVE3103.

15. A method according to Claim 12, wherein the *Agrobacterium* cells are capable of targeting wounded regions in the plant.

16. A method according to Claim 15, wherein the *Agrobacterium* is *A. tumefaciens* strains LBA4404 and C58.

17. A method according to Claims 12 to 16, wherein during the incubation of the *Lemnaceae* plant tissue with the *Agrobacterium* cells vacuum infiltration is applied.

18. A method according to Claim 12, wherein prior to incubation of the *Lemnaceae* plant tissue with the *Agrobacterium* cells the plant's meristematic zone is exposed by removal of the daughter fronds.

19. A method for the genetic transformation of a plant comprising: cutting the plant into particles of a size such that they still contain undamaged meristematic tissue capable of developing into full plants; incubating said particles with *Agrobacterium* cells containing transforming DNA molecules, whereby said transforming DNA is introduced into meristematic cells in said particles; and producing transformed plants from the transformed meristematic tissue.

20. A method according to Claim 19, wherein the plant is a *Lemnaceae* plant.

21. A method according to Claim 19 or 20, wherein the particles have an average diameter above about 150 μm.

22. A method according to Claim **21**, wherein the particles have an average diameter of about 150 μm - 750 μm.

23. A method for the stable genetic transformation of a *Lemnaceae* plant comprising microinjecting *Agrobacterium* cells containing a transforming DNA into the meristematic zone of the plant, whereby the meristemic tissue becomes stably transformed with said DNA.

24. A method according to Claim 23, carried out *in planta*.

25. A method for the *in planta* transformation of *Lemnaceae* plants comprising:

i. exposing the plant's meristematic zone by removal of the daughter fronds;

ii. incubating the plant with *Agrobacterium* cells capable of targeting to the meristemic tissue.

26. A method according to Claim 25, wherein the *Agrobacterium* cells are *A. tumefaciens* strains EHA105, EHA101 and GVE3103.

27. A method according to any one of Claims 12 to 26, wherein the *Agrobacterium* cells are brought into contact, prior or during the transformation method, with a booster medium capable of enhancing the *Agrobacterium* cell's virulence.

28. A method according to any one of Claims 12 to 26 wherein the transformation process takes place in a media having a pH below about 5.2.

29. A method according to Claim 28, wherein the booster medium further comprises a fresh cell suspension obtained from a dicotyledonous plant.

30. A method according to Claims 28 or 29, wherein the fresh cell suspension is at a concentration of 1-10% (w/v).

31. A method according to Claims 28 to 30, further comprising caffeine at a concentration of 100-500 mg per liter of medium.

32. A method according to any one of Claims 28 to 31, wherein the fresh cell suspension of a dicotyledonous plant is obtained from the family of *Solanaceae*.

33. A method according to any one of Claims 26 to 32, wherein the medium is a plant culture medium having a pH of about 3.5 to 4.2, and comprising 1-10% (w/v) of fresh cell suspension of *Nicotiana tabacum* and 100-500 mg per liter of medium caffeine.

34. A method according to Claim 27, wherein the booster medium comprises a *Lemnaceae* plant extract.

35. A method according to Claim 34, wherein the *Lemnaceae* plant extracts are *Spirodela punctata* extracts.

36. A transformed *Lemnaceae* plant obtained by the method of any one of Claims 12 to 35.

37. A booster medium for enhancing *Agrobacterium* cell's virulence comprising plant tissue culture at a pH below about 5.2.

38. A booster medium according to Claim 37, further comprising a fresh cell suspension of a dicotyledonous plant.

39. A booster medium according to Claim 38, wherein the fresh cell suspension is at a concentration of 1-10% (w/v).

40. A booster medium according to any of Claims 37 to 39, further comprising caffeine at a concentration of 100-500 mg per liter of medium.

41. A booster medium according to any of Claims 37 to 40, wherein the fresh cell suspension is of plants from the family of *Solanaceae*.

42. A booster medium according to any of Claim 37 to 41, comprising plant growth medium at a pH of above 3.5 to 4.2, 1-10% (w/v) of fresh cell suspension of *Nicotiana tabacum*, and 100-500 mg per liter of medium caffeine.

43. A booster medium for enhancing *Agrobacterium* cell's virulence comprising an extract from *Lemnaceae* plants.

44. A booster medium according to Claim 41, comprising extracts of *Spirodela punctata* plants.

45. A method for maintaining morphogenetic *Lemnaceae* calli for long-periods of time comprising culturing the calli in a medium having a low level of sucrose.

46. A method according to Claim 45, wherein the sucrose level is less than 1.5%.

47. A method for the regeneration of plants from calli wherein the plant's growth medium has sucrose levels below 1.5% and comprises: B5, minerals and organic compounds.

48. A method for the production of highly regenerative calli, wherein the calli's growth medium has sucrose levels below 1.5% and comprises B5, minerals and organic compounds.

49. A method according to Claim 47 or 48, wherein the growth media further comprises phytohormones.

50. A method for the production of highly regenerative calli, wherein the calli's growth medium has sucrose levels below 1.5% and comprises B5, minerals, organic compounds and selection agents.

51. A method according to Claim 50, wherein the selection agents are selected from the group consisting of: antibiotics, herbicides, or metabolic inhibitors.

52. A method for the production of stable transformed plants, wherein the growth media has sucrose levels below 1.5% and comprises B5, minerals and organic compounds.

53. A method according to Claim 52, wherein the growth media further comprises phytohormones.

54. A method of production of a product of interest, comprising growing a transformed *Lemnaceae* according to one of claims 1 to 8, coding said product in an appropriate culture medium, under conditions enabling the production of said product of interest.

55. The method as claimed in claim 54, wherein the product of interest is further isolated and purified.

56. A method as claimed in one of claims 54 or 55, wherein the product of interest is a chemical or a biological product.

57. A method as claimed in claim 56, wherein the product of interest is selected from the group consisting of polypeptides, proteins, carbohydrates, lipids, alkaloids, pigments or vitamins.

AMENDED CLAIMS

[received by the International Bureau on 5 March 1999 (05.03.99); new claims 58-61 added; remaining claims unchanged (1 page)]

52. A method for the production of stable transformed plants, wherein the growth media has sucrose levels below 1.5% and comprises B5, minerals and organic compounds.

53. A method according to Claim 52, wherein the growth media further comprises phytohormones.

54. A method of production of a product of interest comprising growing a transformed *Lemnaceae* according to one of Claims 1 to 8, coding said product in an appropriate culture medium, under conditions enabling the production of said product of interest.

55. The method as claimed in Claim 54, wherein the product of interest is further isolated and purified.

56. A method as claimed in one of Claims 54 or 55, wherein the product of interest is a chemical or a biological product.

57. A method as claimed in Claim 56, wherein the product of interest is selected from the group consisting of polypeptides, proteins, carbohydrates, lipids, alkaloids, pigments or vitamins.

58. A method according to Claim 35, wherein the *Lemnaceae* is *Spirodela*.

59. A method for forming *Lemnaceae* calli by separating between the mother frond and the daughter frond, using a pinching motion.

60. A method according to Claim 47, wherein the plants are *Lemnaceae*.

61. A method according to Claim 60, wherein the plants are *Spirodela*.

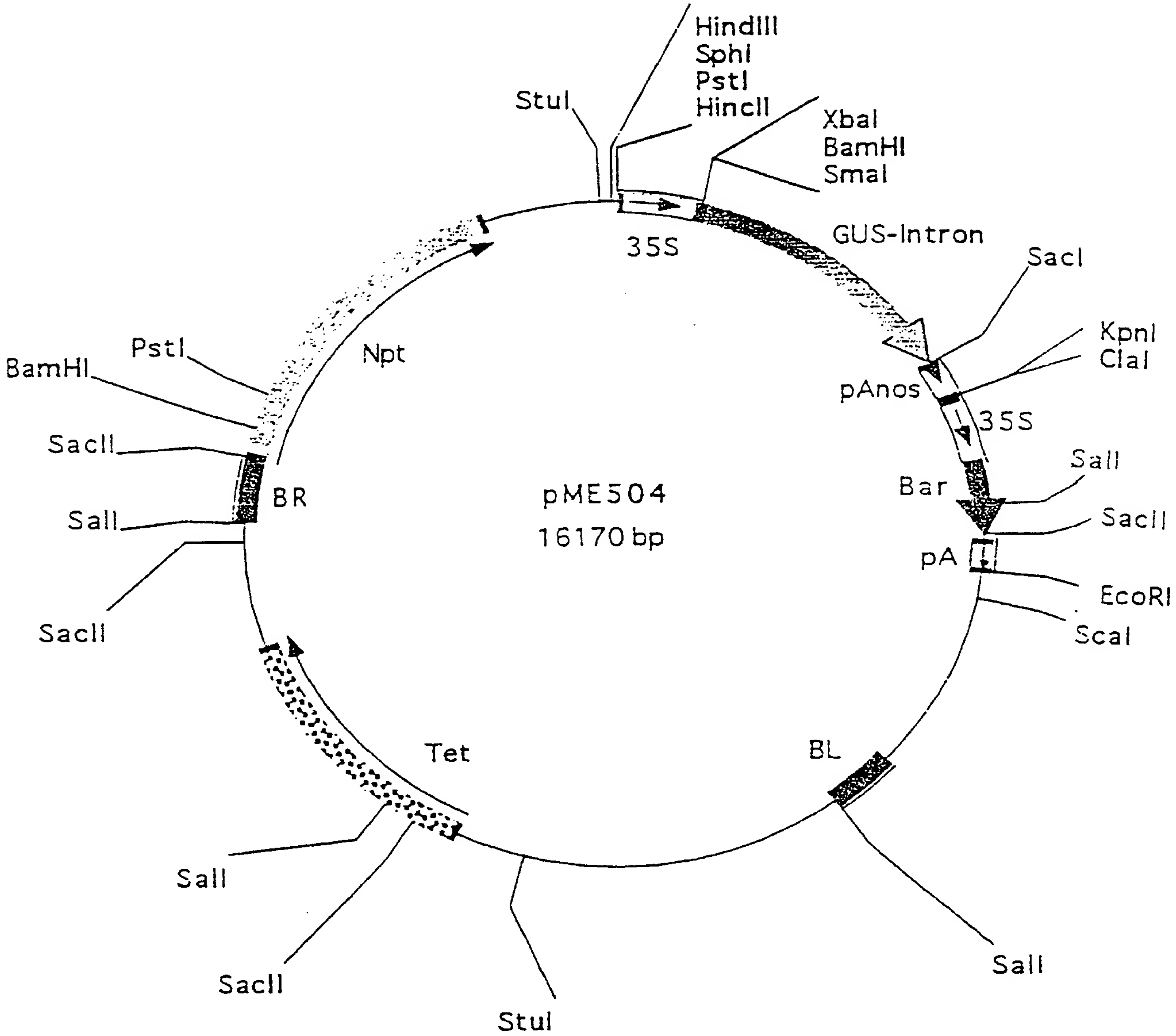
HindIII
SphI
PstI
HincII
StuI
XbaI
BamHI
SmaI
GUS-Intron
35S
SacI
KpnI
ClaI
pAnos
35S
SalI
Bar
SacII
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Figure 1

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IL 98/00487

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 C12N5/04 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FREY M. ET AL.: "Evidence for uptake of plasmid DNA into intact plants (Lemna perpusilla) proved by an E. coli transformation assay" ZEITSCHRIFT FÜR NATURFORSCHUNG, vol. 35c, 1980, pages 1104-1106, XP002067607 see the whole document ---	1,2
X	WO 89 12102 A (TEXAS A & M UNIVERSITY SYST) 14 December 1989 * see esp. p. 6 l. 25 ff. * ---	19,21,22
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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search 6 January 1999	Date of mailing of the international search report 13/01/1999
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Kania, T

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

In[illegible]Application No
PCT/IL 98/00487

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A	CHANG W. AND HSING Y.: "Callus formation and regeneration of frond-like structures in Lemna perpusilla 6746 on a defined medium" PLANT SCIENCE LETTERS, vol. 13, 1978, pages 133-136, XP002067611 cited in the application see the whole document ---	1-57
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INTERNATIONAL SEARCH REPORT

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